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A STUDY ON AUTOLYSIS OF ANIMAL TISSUES.

By KARL GUSTAV DERNBY.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

(Received for publication, May 1, 1918.)

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INTRODUCTION.

By autolysis we mean the non-bacterial postmortem self-decomposition of animal or plant tissues. Salkowski (1890) first recognized the phenomenon as due to enzymatic action, and called it "autodigestion." The name "autolysis" was introduced later by Jacobi (1900). The many papers published on autolysis have pursued two different lines of investigation, one concerning the products of autolysis, the other concerning the action and nature of the proteolytic enzymes causing the autolysis.

Through the work of authors such as Salkowski, E. Fischer, Wroblewski, Kossel, Kutscher, Levene, and others on the problem of the products of autolysis, we know that the end-products of tissue decomposition are amino-acids and intermediate products, albumoses, peptones, and peptides, such as are obtained by the action of pepsin, trypsin, and erepsin. But the nature and manner of action of the different proteolytic enzymes is still a matter of discussion, and the question is one of biological importance, because it appears probable that these autolytic enzymes are not postmortem products, but the very enzymes which act during the life of the cells.

The literature concerning the mode of action of these enzymes is a maze of contradictions. The most exhaustively studied factor is the influence exerted by acids and bases on autolysis. Early investigators as Schwiening (1894) and Biondi (1896) stated that alkalies check and acids facilitate autolysis. Hedin and Rowland (1901) demonstrated the presence of proteases in the press juices of animal tissues, obtained by the Buchner method, and state that alkaline reaction depresses autolysis, and that acids in weak concentrations facilitate it, the rate of autolysis being greatest in the presence of 0.25 per cent acetic acid. Hedin (1906) especially called attention to what he termed "α-protease," a proteolytic enzyme acting in alkaline solution and distinctly different from "β-protease," which acts only in acid reaction. Hedin also made the interesting observation, that if a tissue such as liver or spleen is pre-treated with dilute acids and then transferred into alkaline solution, the rate of autolysis in alkaline medium is much greater than if the tissue is immersed in the alkaline solution without preliminary acid treatment. In order to explain this phenomenon he assumes the existence of "antiproteases," which inhibit only the α-protease, and are readily destroyed in acid solution.

A similar theory for the autolysis of yeast is proposed by Buchner and Hahn (1909, 1910) who assume that there are "antiproteases, probably of lipoid character," which protect the proteins from being destroyed by the proteolytic enzymes. Satisfactory proof of the existence of such antienzymes, seems, however, to be lacking.

Other authors besides Hedin have studied the antiproteolytic actions of serum and different tissues; *e.g.*, Weinland (1903), Landsteiner (1900), and Opie (1905, 1906). Attention may also be called to the work of Levene and Stookey (1903) in this respect. Levene (1905) has given an exhaustive review of all data concerning autolysis, known up to that date. As has been said, most authors have found that alkalies inhibit autolysis, but there are others such as Baer and Loeb (1905) who state that considerable autolysis occurs in alkaline medium. Opie (1905, 1906) and later Dochez (1910) have published a series of papers concerning autolysis of various organs. Dochez contributes valuable data on liver autolysis, confirming the observation of Hedin and others, and gives a good historical review of the field.

More recently some American authors, especially Bradley (1915, 1916) and collaborators and Morse (1917) have published papers on autolysis, and on the influence thereon of the reaction of the medium. Morse assumes autolysis to be an "autocatalytic" reaction, due to the spontaneous increase in the hydrogen ion concentration. Bradley explains the accelerating influence of acid upon autolysis by assuming that the increased hydrogen ion concentration alters the proteins into forms more readily digested by the enzymes. Bradley also makes the statement, that there probably is no "latent period" in the autolysis, and that the first stage of autolysis is the formation of peptones and peptides, amino-acids appearing only later.

As far as the author knows, however, there have been no other attempts made with modern methods to study the influence of the hydrogen ion concentration on autolysis of animal tissues.

In his classical work Sörensen (1909) emphasizes that it is quite as important to determine the hydrogen ion concentration and its eventual changes as the temperature of biochemical processes. Thanks to him we now have accurate methods to measure hydrogen ion concentration. In this connection attention must be called to the theory, first suggested by Loeb and later adopted by Michaelis (1914), that the enzymes in fact are ampholytes and that enzymatic action is a dissociation phenomenon. Ringer (1916) does not accept this theory.

In a previous paper the author has studied the proteolytic enzymes in yeast cells and the effect of hydrogen ion concentra-

tion on them. Briefly stated this problem has been studied in the following manner. Buchner and Hahn (1903) who discovered cell-free fermentation, also studied the proteolytic activity of the press juice, and claimed that this contained an enzyme of trypsin type, which, however, acted only in acid solution, and to which they gave the name "endotryptase." They described it as acting best in the presence of 0.2 per cent hydrochloric acid. There have been a great number of papers dealing with this problem, mostly confirming the work of Hahn. The English botanist, Vines (1904, 1909), however, claims that in the yeast cells there are two different kinds of proteolytic enzymes, one of pepsin, and one of trypsin type, and that they differ in solubility in sodium chloride solution.

The writer (1917) has been able to show, that in yeast cells there must be at least three different groups of proteolytic enzymes, acting at different hydrogen ion concentrations (Fig. 1).

	Action.	Optimum hydrogen ion concentration.
		pH
Pepsin	Splitting proteins into peptones.	About 4
Tryptase	" peptones " peptides.	7.0
Ereptase	" peptides " amino-acids.	7.8

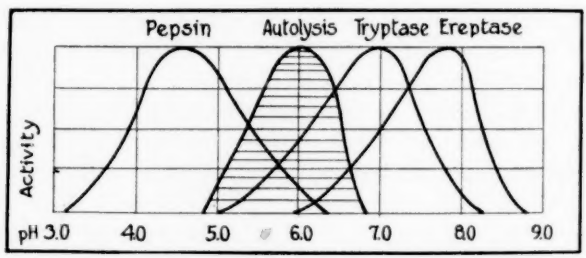

FIG. 1.

The optimum for the action of pepsin lies in the acid region, that for the tryptase almost at the neutral point and that for the ereptase in alkaline solution. The optimum for autolysis (the maximum decomposition of the protein) at constant hydrogen ion concentration is however at pH = 6, between the optima of pepsin and tryptase, and at the point where the two enzymes can act simultaneously. The extent of autolysis is shown in the shaded portion of the figure (Fig. 1).

In the following the author has extended the investigations to different tissues of animal origin, always controlling the hydrogen ion concentration of the medium and its changes.

General Methods.

Preparation of Tissue Suspensions.—The tissues used in the following experiments were obtained from freshly killed normal animals, dogs or pigs. The liver, pancreas, and spleen tissues, weighing from 50 to 300 gm. and freed mechanically from attaching fat, were minced in a meat chopper with an ordinary cutter, repeatedly washed with 1 per cent sodium chloride solution to remove most of the blood, and filtered through cheese-cloth. The remaining mash was then minced with a "nut butter cutter" until it became a very fine homogeneous suspension which could be easily pipetted and contained about twice as much water as the original tissue. How much of these suspensions was used in each experiment, and their content of nitrogen is noted under each experiment. These suspensions were kept in the ice box, and toluene and chloroform in sufficient quantities were added to prevent bacterial growth. All the digestions were carried out in an incubator at 37°C.

Nitrogen Determinations.—The chemical methods used for showing the progress of the enzymatic reactions were the following. The total amount of nitrogen in the solutions was determined by the Kjeldahl method. The coagulable protein was precipitated with 2.5 per cent trichloroacetic acid (9 parts of acid to 1 part of solution). After removing the coagulum the remaining nitrogen in an aliquot part of the filtrate was determined by the Kjeldahl method. The amount of amino nitrogen was determined by the Van Slyke method[1] in the solution after removing the solid particles by filtration. The peptide nitrogen, that is the nitrogen from the $-CO-NH-$ group of albumoses, peptides and peptones, was determined by

[1] In an earlier paper the author used Sörensen's formol titration for determining the amount of amino nitrogen liberated. This method measures practically all NH_2 groups, but it requires rather large amounts of solution for each test to get accurate values. The Van Slyke method does not give the accurate amount of nitrogen for some substances, *e.g.* ammonia and glycyl derivatives, but has the great advantage that carbon dioxide and phosphates do not interfere with it, and rather small amounts of solutions are required for each test.

hydrolyzing the solutions with hydrochloric acid. 1 part of concentrated hydrochloric acid was added to 1 part of solution. The mixture was heated for 24 hours at 100°, concentrated in a vacuum to remove the acid, and finally neutralized with sodium hydroxide and made up to the original volume of the amino-acid solution. Then the increase in amino nitrogen was determined by the Van Slyke method. The free ammonia was determined by addition of potassium carbonate and aeration into 0.02 M hydrochloric acid.

Determination of the Hydrogen Ion Concentration.—The hydrogen ion concentrations in all the experiments were determined by the colorimetric method of Sörensen with the indicators of Clark and Lubs (1917). Because of their small "protein error" these new indicators are well fitted for solutions containing protein substances. To express the value of the hydrogen ion concentration Sörensen's symbol pH is used. pH indicates the negative logarithm of the hydrogen ion concentration in terms of normality.

The standard solutions were made up of pure chemicals, and their purity was controlled by the electrometric method. The following solutions were used:

$\frac{1}{10}$ M hydrochloric acid.
$\frac{1}{10}$ " sodium citrate.
$\frac{1}{15}$ " potassium dihydrogen phosphate, KH_2PO_4.
$\frac{1}{15}$ " disodium hydrogen phosphate, Na_2HPO_4, $2H_2O$.

The last two salts were Kahlbaum's preparations after Sörensen. By means of mixtures of these solutions any hydrogen ion concentration between pH = 1.0 and pH = 9.0 can be reproduced, and they cover practically the whole range of the hydrogen ion concentration which can be considered in the study of autolysis.

The indicators used have been the following.

	Range of pH.
Thymolsulfonephthalein (thymol blue)	1.2–2.8
Tetrabromophenolsulfonephthalein	3.0–4.6
Methyl red	4.4–6.0
Dibromo-*o*-cresolsulfonephthalein	5.2–6.8
Dibromothymolsulfonephthalein	6.0–7.6
Phenolsulfonephthalein	6.8–8.4
o-Cresolsulfonephthalein	7.2–8.8
Thymolsulfonephthalein	8.0–9.6
o-Cresolphthalein	8.2–9.8

As most of the solutions examined were more or less colored and turbid, they have been diluted two or three times before the examination. According to Clark such a dilution does not essentially alter the hydrogen ion concentration of the solutions. Clark also suggests the use of a special comparator rack for colored solutions. It has been used here in a slightly modified shape and found to work excellently. In another paper is a complete description of it (Avery and Dernby, 1918). The method is fairly accurate; the experimental error averages less than 0.1 in pH.

For the identification of the different proteolytic enzymes in the tissues the following two methods have proved to give the best results, and to be the most convenient to carry out.

Gelatin Method.—The method of Fermi (1891, 1906) of liquefying gelatin in the form suggested by Palitzsch and Walbum (1912) has been used to demonstrate the presence of pepsin-like enzymes. In some cases also enzymes of trypsin type attack gelatin. As this method does not seem to be generally known, its main features may be described here. 700 gm. of gelatin are dissolved in 1,250 cc. of hot water, the solution is passed through cheese-cloth, and 2 gm. of thymol suspended in water are added. The solution is then diluted to 2 liters and kept in stock in an ice box. As used in the following experiments it contained 37.5 mg. of nitrogen per 1 cc.

Before the experiments, 200 gm. of the stock solution were taken and diluted to 500 cc. For most of the experiments 15 cc. of this solution were used, hydrochloric acid, sodium hydroxide, phosphates, and tissue suspension added, and the whole was diluted to 30 cc. The solutions were kept in small Erlenmeyer flasks, covered with an inverted beaker, and all the digestions took place in an incubator at 37°. After certain intervals 5 cc. from each flask were removed and pipetted into a thin test-tube, which subsequently was dipped into ice water and cooled for exactly 15 minutes. Acids in less than M concentration do not interfere much with the liquefaction of gelatin, but even comparatively small amounts of alkalies do. Therefore the alkaline solutions must be neutralized by addition of a calculated amount of acid before samples are removed for cooling. After 15 minutes the tubes were removed from the ice bath, and the degree of liquefaction was estimated.

In order to make possible a graphic representation of the results in curves and to facilitate their presentation, the estimate is expressed in numbers. Palitzsch and Walbum utilize twelve numbers, out of which, however, in this paper only seven are used. They are the following:

0 = Completely solid.
1 = Solid, but small pieces may be torn off by strong shaking.
2 = " " the surface moves somewhat when tubes are skaken.
3 = Soft.
4 = Half liquid.
5 = Almost "
6 = Entirely "

Peptone Method.—For the demonstration of trypsin- or erepsin-like enzymes solutions of Witte's peptone have been used. A 4 per cent solution of Witte's peptone was made up (Dernby, 1917) and used as stock solution. As used here it contained 7.92 mg. of total nitrogen per 1 cc. In order to carry out the digestions with different tissues under identical conditions seven different mixtures of peptone, phosphates, sodium hydroxide or hydrochloric acid, and water were prepared beforehand, all possessing different hydrogen ion concentrations. The following scheme shows the composition of the mixtures and the resulting hydrogen ion concentration.

40 cc. 4 per cent peptone solution + 10 cc. 0.5 M phosphates + HCl or NaOH + water = 180 cc.

No.	0.1 N HCl.	0.1 N NaOH.	0.5 M phosphates.		pH
			KH_2PO_4	Na_2HPO_4	
	cc.	cc.	cc.	cc.	
1	16.0	—	10.0	0	4.0
2	8.0	—	9.0	1.0	5.2
3	3.0	—	5.0	5.0	6.8
4	—	—	2.0	8.0	7.3
5	—	3.0	1.0	9.0	7.7
6	—	8.0	0.5	9.5	8.0
7	—	14.0	0	10.0	8.5

An experiment was carried out in the following manner. In seven test-tubes were transferred 9 cc. of each of these solutions, and to each of them was then added 1 cc. of the enzyme prepa-

ration or tissue suspension. Care must be taken that the tissue suspension does not essentially alter the hydrogen ion concentration of the solutions. In some cases a few drops of hydrochloric acid or sodium hydroxide had to be added beforehand in order to keep the initial hydrogen ion concentration at the desired point.

At the beginning and after certain intervals the amino nitrogen and the hydrogen ion concentration were determined as described above.

Artificial Autolysis.

In explanation of the autolysis of yeast we have suggested that there are enzymes in the cells of peptic, tryptic, and ereptic character, all of them working at different hydrogen ion concentrations, and "autolysis" is the resultant of the action of these different enzymes. To test this theory more completely it appears desirable to carry out an "artificial autolysis," that is to treat a known protein substance with a mixture of pepsin and trypsin, and analyze the reaction products after digestion at different hydrogen ion concentrations. In order to do this the following experiment was carried out by means of the method of Palitzsch and Walbum (1912) with gelatin as substrate. The gelatin solutions prepared as above were treated with certain amounts of hydrochloric acid or sodium hydroxide in order to obtain a wide range of different hydrogen ion concentrations. A solution containing both pepsin and trypsin was made by dissolving a 0.1 gm. portion of each enzyme (Fairchild) in 50 cc. of distilled water. Of this solution 1.0 cc. was added to 30 cc. of gelatin mixture, No buffer mixture was used, the initial hydrogen ion concentration was measured as stated, and if it changed a little during the digestion it was of no essential importance. Table I and Fig. 2 show the result.

The gelatin is liquefied in two regions, one on the acid side, the other on the alkaline. There are two well defined optima, one at about pH = 3 and the other at about pH = 8. It is obvious that the first optimum must be due to the action of the pepsin, the other to the action of the trypsin.

In the above experiment we have used a substrate, gelatin, which is attacked by both pepsin and trypsin. Most native

animal proteins, are attacked by pepsin but not by trypsin, the latter enzyme acting only on the products of peptic digestion. We must expect results quite different from the above when a substrate not attacked directly by trypsin is acted on by the two enzymes.

Table II gives an experiment where boiled pig's liver, thoroughly minced and washed, is used as such a substrate.

TABLE I.

Trypsin and Pepsin on Gelatin.

15 cc. gelatin + HCl or NaOH + 1.0 cc. enzyme solution + water = 30 cc.

Enzyme solution: 0.1 gm. pepsin (Fairchild) + 0.1 gm. trypsin (Fairchild) + 50 cc. water.

Tube No.	Added.		Initial pH.	Degree of liquefaction after digestion at 37° for				
	HCl	NaOH		30 min.	60 min.	120 min.	180 min.	360 min.
1	6.0 cc. N	—	2.0	1½	1½	2	2½	3
2	4.0 " "	—	2.5	1½	3	3½	4	5
3	3.0 " "	—	3.1	1	3	3½	4	5
4	1.6 " "	—	3.5	½	2	2½	3	4
5	6.0 " 0.1 N	—	4.1	0	½	1	2	5
6	—	—	4.9	0	0	0	1	6
7	—	3.0 cc. 0.1 N	6.0	0	0	1	3	6
8	—	4.0 " 0.1 "	6.8	0	1	2	5	6
9	—	6.0 " 0.1 "	7.3	0	2	4	6	6
10	—	7.0 " 0.1 "	7.7	½	3	5	6	6
11	—	8.0 " 0.1 "	8.1	½	3	5	6	6
12	—	1.5 " N	8.6	½	2	4	6	6

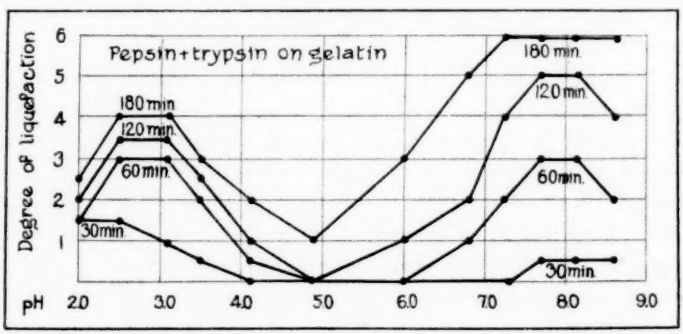

FIG. 2.

The optimum for the reaction is about pH = 5 to 6. This pH lies between the optima of pepsin and trypsin, but is where both enzymes can act simultaneously. On the alkaline side of the optimum there has been more digestion than on the acid side, presumably because the liver contains some proteins digestible directly by trypsin. These results are by no means to be regarded as complete evidence for the general theory of autolysis developed in the following, but they prove that it is easy by known enzymes and substrates to reproduce a process resembling autolysis.

TABLE II.

Trypsin and Pepsin on Liver Proteins.

2 cc. boiled liver suspension + 1 cc. 0.5 N phosphates + HCl or NaOH + 1 cc. enzyme + water = 10 cc.

2 cc. suspension contained 23.6 mg. total nitrogen.

Enzyme solution = 0.1 gm. pepsin (Fairchild) + 0.1 gm. trypsin in 50 cc. water.

Digestion continued 12 hours at 37°.

Tube No.	Initial pH.	Increase in amino nitrogen.
		mg.
1	About 2	1.55
2	3.5	1.67
3	4.5	1.70
4	5.5	2.80
5	6.4	3.20
6	7.0	2.76
7	7.5	2.68
8	8.0	2.46

Identification of the Enzymes.

The classification of proteolytic enzymes hitherto used is made on three lines. (1) The substrate which they attack. (2) The products yielded. (3) The optimum hydrogen ion concentration for the enzyme action.

As the classical types for the proteolytic enzymes are the three excreted enzymes from the animal body, the pepsin, the trypsin, and the erepsin, the scheme for their actions is the following:

Enzyme.	Substrate.	Products.	Optimum pH.
Pepsin	Native proteins.	Peptones.	1.5
Trypsin	Albumoses, peptones, and some native proteins, such as casein and fibrin.	Peptides, amino-acids.	7.8
Erepsin	Peptides.	Amino-acids.	7.8

Since it is difficult, if not impossible, to separate the proteolytic enzymes from the tissue cells, a suspension of very finely minced tissue is used in the following as enzyme preparation. As substrates gelatin solutions have been used for detecting enzymes of pepsin type, and peptone solutions for detecting enzymes of trypsin or erepsin type. Briefly, those two tests are called the "gelatin test" and the "peptone test."

Yeast Cells.—In an earlier paper it is shown that the existence of both pepsin- and trypsin-like enzymes can be easily demonstrated by the action of yeast juice on gelatin (Dernby, 1917). Such an experiment is repeated here, and Table III and Fig. 3 show the result.

TABLE III.

Yeast Juice on Gelatin.

15 cc. gelatin + HCl or NaOH + water + 0.5 cc. yeast juice = 30 cc.

Tube No.	Added.		Initial pH.	Degree of liquefaction after digestion at 37° for			
	HCl	NaOH		1 hr.	3 hrs.	6 hrs.	24 hrs.
1	26.0 cc. 0.1 N	—	3.2	1	2½	4	4
2	16.0 " 0.1 "	—	3.5	1½	4	5	5
3	10.0 " 0.1 "	—	3.7	1½	3½	5	6
4	6.0 " 0.1 "	—	4.0	½	2	3½	6
5	—	—	4.5	0	2	3	6
6	—	1.9 cc. 0.1 N	5.1	0	3	6	6
7	—	3.0 " 0.1 "	5.8	1	5	6	6
8	—	4.0 " 0.1 "	6.7	1½	6	6	6
9	—	5.0 " 0.1 "	7.2	2	6	6	6
10	—	6.0 " 0.1 "	7.9	0	4	6	6

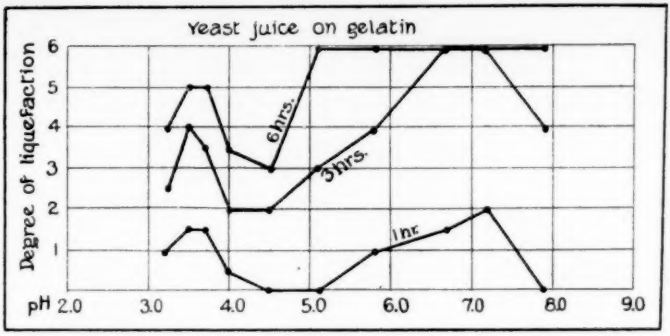

FIG. 3.

Two optima are seen, one at pH = 3.5, corresponding to that of gastric pepsin, the other at pH = 7, corresponding to that of animal trypsin.

Liver.—Watery extracts obtained by grinding liver with sand were made in various manners, using fresh and half autolyzed liver, and extracts made with water, weak acids, and alkalies; but the extracts, which always contained much protein, attacked neither gelatin nor peptones. This was also true for spleen and confirms the statements of preceding authors; *e.g.*, Levene. On the other hand, if a little of the liver suspension was mixed with gelatin, the gelatin was liquefied, and preliminary tests showed that this reaction took place only in acid solution. This reaction was first discovered by Theobald Smith (1894).

Tables IV and V give the results of two similar experiments. In Fig. 4 the results from Table V are graphically reproduced.

Tables IV and V show that the proteolytic action on gelatin takes place only in acid solution, and the optimum hydrogen ion concentration is between pH = 3 and pH = 4. Several similar experiments gave the same result, and it must especially be noted that in no case was any proteolytic action whatsoever obtained on the alkaline side. In this respect the liver and spleen, stomach cells, and leukocytes differ from pancreas and yeast cells.

Tryptase.—Preliminary experiments showed that liver suspension readily attacked a solution of Witte's peptone, and that the rate of reaction was greatest in weakly alkaline solution. The

TABLE IV.

Liver Suspension on Gelatin.

15 cc. gelatin + HCl or NaOH + 1 cc. liver suspension (fresh) + water = 30 cc.

1 cc. liver suspension contained 12.0 mg. total nitrogen.

Tube No.	Added.		Initial pH.	Degree of liquefaction after digestion at 37° for				
	HCl	NaOH		1 hr.	3 hrs.	6 hrs.	12 hrs.	24 hrs.
1	4.0 cc. N	—	2.6	0	0	0	0	0
2	2.6 " "	—	3.0	0	0	0	1	2
3	1.6 " "	—	3.4	1	2	3	4	6
4	10.0 " 0.1 N	—	3.8	0	1	2	3	5
5	6.0 " 0.1 "	—	4.4	0	0	1	2	4
6	—	—	5.1	0	0	0	1	3
7	—	1.8 cc. 0.1 N	5.8	0	0	0	0	1
8	—	3.0 " 0.1 "	6.8	0	0	0	0	0
9	—	4.0 " 0.1 "	7.3	0	0	0	0	0
10	—	6.0 " 0.1 "	7.8	0	0	0	0	0

TABLE V.

Liver Suspension on Gelatin.

15 cc. gelatin + HCl or NaOH + 1 cc. liver suspension (4 days in ice box) + water = 30 cc.

1 cc. liver suspension contained **22.6** mg. total nitrogen.

Tube No.	Added.		Initial pH.	Degree of liquefaction after digestion at 37° for		
	HCl	NaOH		1 hr.	2 hrs.	3 hrs.
1	2.6 cc. N	—	3.2	0	2	3
2	1.6 " "	—	3.5	2	$3\frac{1}{2}$	5
3	10.0 " 0.1 N	—	3.7	3	$3\frac{1}{2}$	5
4	6.0 " 0.1"	—	4.0	2	$2\frac{1}{2}$	4
5	—	—	4.5	1	1	3
6	—	1.8 cc. 0.1 N	5.1	0	1	2
7	—	3.0 " 0.1 "	5.8	0	0	0
8	—	4.0 " 0.1 "	6.7	0	0	0
9	—	5.0 " 0.1 "	7.2	0	0	0

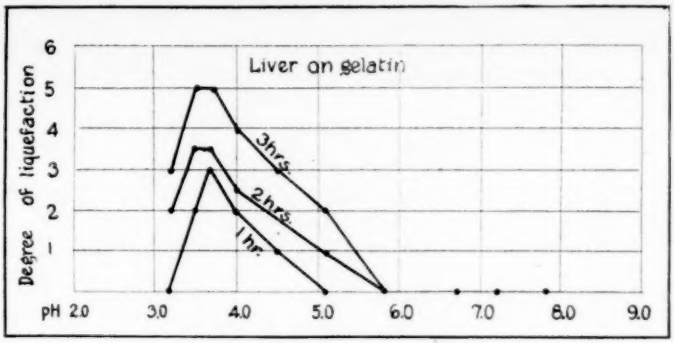

FIG. 4.

objection may be raised that when peptone solution is mixed with such large amounts of liver suspension the amount of total nitrogen of the liver exceeds that of the peptone. One cannot tell

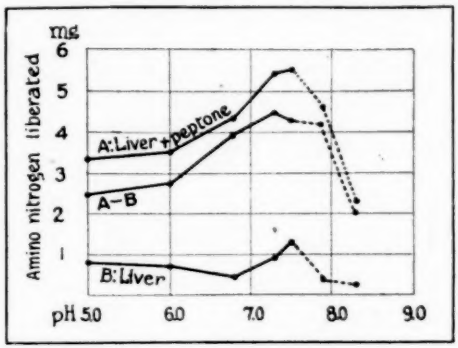

FIG. 5.

whether the amino nitrogen formed originates from the peptone or from the liver tissue. Table VI shows also a control experiment without any peptone, which indicates that comparatively small amounts of amino nitrogen are formed from the liver tissue alone. In Fig. 5 the results are represented graphically.

A considerable increase in the hydrogen ion concentration occurred during the digestions in spite of the presence of the phosphates. This was presumably due to the formation of acids from

liver glycogen. Greater concentration of buffer salts to reduce the change did not appear advisable because of the effect of higher concentrations of salts in checking enzyme action.

TABLE VI.

Liver Suspension on Peptone.

A: 9 cc. peptone solution + 1 cc. liver suspension (7 days in ice box) + 1 cc. 0.5 M phosphates = 10 cc.

B: 1 cc. liver suspension + 1 cc. 0.5 M phosphates + HCl or NaOH + water = 10 cc.

1 cc. liver suspension contained 22.60 mg. total nitrogen.
1 " " " " 2.90 " amino "
9 cc. peptone solution " 15.84 " total "
9 " " " " 1.82 " amino "

Tube No.	pH			Increase in amino nitrogen after 48 hrs. at 37°.		
	Initial.	After digestion, A.	After digestion, B.	A	B	A − B (amino nitrogen formed from peptone).
				mg.	*mg.*	*mg.*
1	5.0	5.0	5.0	3.34	0.85	2.49
2	6.0	5.8	5.7	3.51	0.73	2.78
3	6.8	6.5	6.5	4.36	0.45	3.91
4	7.3	6.9	6.8	5.48	0.98	4.50
5	7.6	7.1	7.1	5.55	1.26	4.29
6	7.9	7.3	7.3	4.62	0.39	4.23
7	8.3	7.4	7.2	2.25	0.23	2.02

The curve for liver digesting alone is noteworthy. At pH = 7.8 there is a marked maximum, at the neutral point there is very little reaction, but in the acid region there seems to be another maximum. This phenomenon will be further discussed under "Autolysis."

The main result obtained from this experiment is that for the digestion of peptone by liver enzymes the optimum pH is about 7.6.

Soluble Erepsase.—Abderhalden and collaborators (1906, 1907, 1916) and others have found that press juice from liver and other tissues has the power to split simple polypeptides into amino-acids. As already mentioned, watery extracts digest neither gelatin nor peptone, but Table VII shows an experiment in which the action of a liver extract on glycyl-glycine has been tried.

TABLE VII.

Liver Extract on Glycyl-Glycine.

5 cc. 0.1 M glycyl-glycine + 2 cc. 0.5 M phosphates + NaOH + 1 cc. liver extract + water = 20 cc.

5 cc. 0.1 M glycyl-glycine contained 14.0 mg. total nitrogen.
1 cc. liver extract " 1.28 " " "

Tube No.	pH		Increased amount of amino nitrogen after 20 hrs. at 37°.
	Initial.	After digestion.	
			mg.
1	5.4	5.4	0.05
2	6.8	6.6	0.90
3	7.1	6.8	1.16
4	7.4	7.1	1.40
5	7.7	7.4	1.62
6	8.0	7.6	1.78
7	8.4	7.7	3.03
8	9.0	7.9	0.0

A slight proteolytic action is evident, and the optimum lies on the alkaline side. It is, however, impossible to determine the optimum exactly because acid-forming substances in the liver cause a great change in the hydrogen ion concentration to the acid side.

Spleen.—Preliminary experiments with spleen suspensions indicated that the proteolytic activities of spleen are analogous to those of liver. Table VIII and Fig. 6 give the result of the gelatin test.

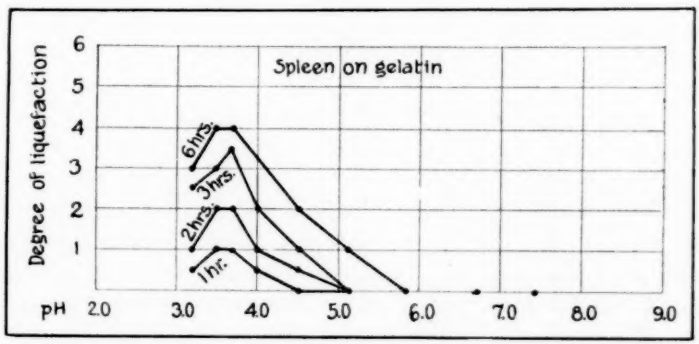

FIG. 6.

TABLE VIII.

Spleen Suspension on Gelatin.

15 cc. gelatin + HCl or NaOH + 1 cc. spleen suspension (2 days in ice box) + water = 30 cc.

1 cc. spleen suspension contained 13.05 mg. total nitrogen.

Tube No.	Added.		pH		Degree of liquefaction after digestion at 37° for				
	HCl	NaOH	Initial.	After 6 hrs. digestion.	1 hr.	2 hrs.	3 hrs.	6 hrs.	20 hrs.
1	2.6 cc. N	—	3.2	3.4	½	1	2½	3	3½
2	1.6 " "	—	3.5	3.6	1	2	3	4	5
3	10.0 " 0.1 N	—	3.7	3.8	1	2	3½	4	5
4	6.0 " 0.1 "	—	4.0	4.1	½	1	2	2½	3
5	—	—	4.5	4.5	0	½	1	2	2
6	—	7.8 cc. 0.1 N	5.1	5.1	0	0	0	1	1
7	—	3.0 " 0.1"	5.8	5.7	0	0	0	0	0
8	—	4.0 " 0.1"	6.7	6.6	0	0	0	0	0
9	—	6.0 " 0.1"	7.4	7.0	0	0	0	0	0

Tryptases.—Spleen tissue is more favorable to deal with than liver because the hydrogen ion concentration does not change so extensively in spleen. Table IX is quite similar to the corresponding Table VI for liver. Fig. 7 gives the results graphically.

TABLE IX.

Spleen Suspension on Peptone.

9 cc. peptone solution + 1 cc. spleen suspension (fresh).
1 " spleen suspension contained 13.05 mg. total nitrogen.
1 " " " " 1.02 " amino "
9 " peptone solution " 15.84 " total "
9 " " " " 1.82 " amino "

Tube No.	pH		Liberated amino nitrogen after 18 hrs. at 37°.
	Initial.	After digestion.	
			mg.
1	6.0	5.9	1.96
2	6.8	6.6	1.92
3	7.3	7.1	2.47
4	7.6	7.4	2.92
5	7.9	7.6	2.64
6	8.3	8.0	0.39

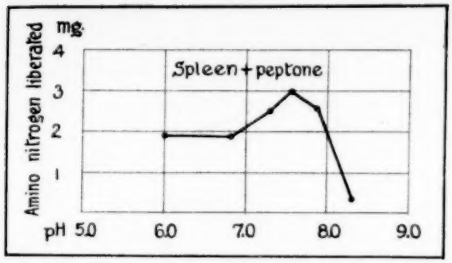

FIG. 7.

Soluble Ereptase.—To see whether there is any ereptase present in a watery extract from spleen the following test was carried out. The extract was prepared in the same way as in the case of liver.

TABLE X.

Spleen Extract on Glycyl-Glycine.

5 cc. 0.1 M glycyl-glycine + 2 cc. 0.5 M phosphates + NaOH + 1 cc. spleen extract + water = 20 cc.

5 cc. 0.1 M glycyl-glycine contained 14.0 mg. total nitrogen.
1 cc. spleen extract " 0.88 " " "

Tube No.	pH		Increased amount of amino nitrogen after 18 hrs. at 37°.
	Initial.	After digestion.	
			mg.
1	5.4	5.4	0.00
2	6.8	6.8	0.28
3	7.1	7.1	0.57
4	7.4	7.4	0.69
5	7.7	7.6	0.80
6	8.0	7.8	0.94
7	8.4	8.1	0.57

The proteolytic action is rather weak and far from a complete decomposition of the peptide, but it is quite evident that there are ereptases present in the extract, and the optimum for their action seems to be about pH = 8.

Pancreas.—The pancreas excretes large amounts of trypsin, which is readily obtained in watery extracts from the tissue. Palitzsch and Walbum have determined the optimum hydrogen on concentration for the action of trypsin on gelatin, and found

the astonishingly high value of pH = 9.1 at 35°. On the other hand Rona and Arnheim (1913) have found the optimum for the tryptic action on Witte's peptone to be at pH = 7.8. Perhaps the liquefaction of gelatin in very alkaline solutions is due in part to other effects than that of the enzymes.

TABLE XI.

Pancreas Extract on Gelatin.

15 cc. gelatin + HCl or NaOH + 3 cc. 0.5 M phosphates + 0.3 cc. pancreas extract + water = 30 cc.

Tube No.	Added.				Initial pH.	Degree of liquefaction after digestion at 37° for				
	HCl	NaOH	Phosphates. $NaHPO_4$	Phosphates. KH_2PO_4		15 min.	30 min.	60 min.	120 min.	20 hrs.
			cc.	cc.						
1	9.0 cc. 0.1 N	—	0	3.0	4.1	0	0	1	2	6
2	—	—	0	3.0	5.0	0	1	3	4	6
3	—	1.0 cc. 0.1 N	0.6	2.4	6.0	0	3	4	5	6
4	—	3.0 " 0.1"	1.5	1.5	6.8	2	4	5	6	6
5	—	4.0 " 0.1"	2.4	0.6	7.3	3	5	6	6	6
6	—	6.0 " 0.1"	2.7	0.3	7.8	4	5	6	6	6
7	—	8.0 " 0.1"	3.0	0	8.2	4	5	6	6	6
8	—	1.4 " N	3.0	0	8.6	3	4	5	6	6
9	—	2.0 " "	3.0	0	9.3	3	4	4	5	6

In Table XI and Fig. 8 is presented an experiment which is a repetition of Palitzsch and Walbum's. Phosphates are used as buffers to keep the hydrogen ion concentration constant. It must be remembered that for measuring the hydrogen ion concentration instead of the electrometric method the colorimetric method with the indicators proposed by Clark has been used. It is a little difficult to locate exactly the optimum but it is evident that it is about or above pH = 8.

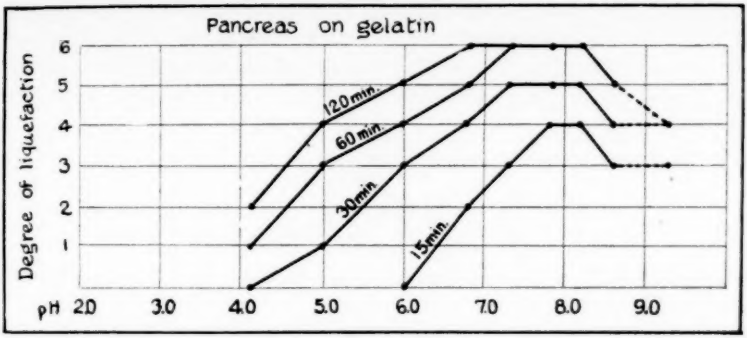

FIG. 8.

Repeated experiments, in which instead of pancreas extracts pancreas suspension was used, indicated that the latter had the power to liquefy gelatin within the limits pH = 3 to pH = 9. The optimum seemed to be in all cases about pH = 8. In the acid region liquefaction was very slow. Only one optimum, the alkaline one, was observed, and there was therefore no evidence of the existence of pepsin-like enzymes. Table XII shows one of these experiments.

TABLE XII.

Pancreas Suspension on Gelatin.

15 cc. gelatin + HCl or NaOH + 1 cc. pancreas suspension +water = 30 cc.

Tube No.	Initial pH.	Degree of liquefaction after digestion at 37° for			
		30 min.	1 hr.	2 hrs.	24 hrs.
1	3.0	0	0	2	6
2	3.3	0	0	4	6
3	3.7	0	1	5	6
4	4.0	0	3	6	6
5	4.5	1	5	6	6
6	5.1	2	6	6	6
7	5.8	4	6	6	6
8	6.8	5	6	6	6
9	7.5	6	6	6	6
10	7.8	6	6	6	6

In the pancreas there is so much trypsin that it may obscure the effects of other enzymes which may be present. The attempt was made therefore to get rid of most of the trypsin by washing the tissue mash thoroughly with distilled water, in order that the residue might be tested for other enzymes. Table XIII and Fig. 9 represent such an experiment.

TABLE XIII.

Pancreas Suspension on Gelatin.

15 cc. gelatin + HCl or NaOH + 1 cc. washed pancreas suspension + water = 30 cc.

Tube No.	Added.		Initial pH.	Degree of liquefaction after digestion at 37° for			
	HCl	NaOH		1 hr.	2 hrs.	3 hrs.	24 hrs.
1	2.6 cc. N	—	3.0	1	2	3	6
2	1.6 " "	—	3.4	2	3	4	6
3	10.0 " 0.1 N	—	3.8	2	2	3	6
4	6.0 " 0.1"	—	4.4	0	0	1	6
5	—	—	5.1	0	0	1	6
6	—	1.8 cc. 0.1 N	5.8	0	1	3	6
7	—	3.0 " 0.1"	6.8	1	2	4	6
8	—	4.0 " 0.1"	7.3	2	3	5	6
9	—	6.0 " 0.1"	7.8	2	4	5	6

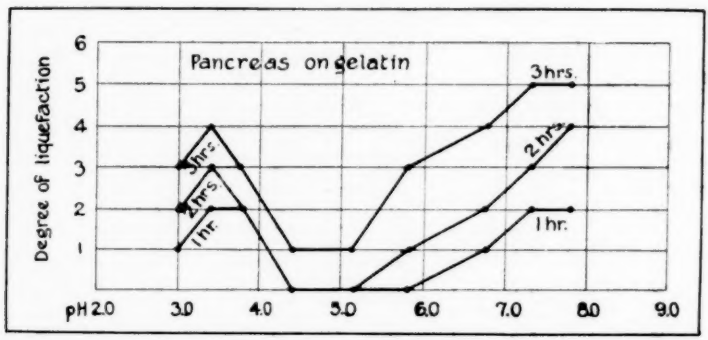

FIG. 9.

Fig. 9 with its two optima is quite analogous to that representing the action of yeast juice on gelatin. It appears that the pancreas contains not only trypsin but also pepsin, the trypsin so

predominating that it must be partially removed before the pepsin can be detected.

TABLE XIV.

Pancreas Extract on Peptone.

9 cc. peptone solution + 1 cc. pancreas extract = 10 cc.
9 " " " contained 15.84 mg. total nitrogen.
9 " " " " 1.82 " amino "
1 " pancreas extract " 3.40 " total "
1 " " " " 0.73 " amino "

Tube No.	Initial pH.	Amino nitrogen liberated after digestion at 37° for	
		2 hrs.	18 hrs.
		mg.	*mg.*
1	5.0	0.94	1.77
2	6.0	0.94	2.41
3	6.8	1.22	3.34
4	7.3	1.66	3.44
5	7.6	1.85	4.16
6	7.9	1.77	4.22
7	8.3	0.77	3.34

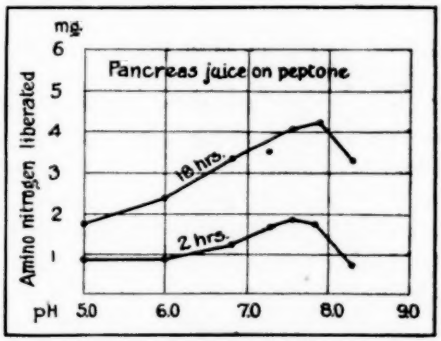

FIG. 10.

Table XIV and Fig. 10 show the action of trypsin on peptone. Here we observe the effects of only the tryptic enzymes, and the experiment confirms the above mentioned observations by Rona and Arnheim (1913), that the optimum for the action of trypsin on peptone is at pH = 7.8.

TABLE XV.

Pancreas Extract on Glycyl-Glycine.

5 cc. 0.1 M glycyl-glycine + 2 cc. 0.5 M phosphates + NaOH + 1 cc. pancreas extract + water = 20 cc.

5 cc. glycyl-glycine contained 14.0 mg. total nitrogen.
1 " pancreas extract " 1.10 " " "

Tube No.	pH		Increased amount of amino nitrogen after 18 hrs. at 37°.
	Initial.	After digestion.	
			mg.
1	5.4	5.4	0.40
2	6.8	6.8	1.60
3	7.1	7.1	2.14
4	7.4	7.5	2.56
5	7.7	7.9	3.02
6	8.0	8.1	2.62
7	8.4	8.4	2.54
8	>9.0	>9.0	0.00

Table XV shows the action of pancreas extract on glycyl-glycine, which is rather easily attacked. The optimum seems to be about pH = 7.9 and is also the same as for the action of erepsin from the intestines on glycyl-glycine. In this respect pancreas extract differs from yeast juice, because the optimum for its action on glycyl-glycine is at pH = 7.8 but the action on peptone is at about pH = 7.0.

Mucous Membrane of the Stomach.—The stomach of a freshly killed dog was cleaned by running water through, the ventricle was opened, and the mucous membrane was removed by means of a piece of glass. About 100 gm. were obtained. Toluene and chloroform were added. Part of the material was ground with sand in a mortar, and the mass afterwards extracted with distilled water. The insoluble parts were filtered off, and the yellowish turbid filtrate was used for the gelatin test.

To the other portion was added 0.5 gm. of powdered calcium carbonate in order to make the mixture neutral, and the mixture was allowed to stand for 24 hours at room temperature. The mash was washed several times by decantation with distilled water in order to remove most of the pepsin. Then the mash was diluted to twice the volume and shaken until a rather uniform suspension was obtained. This suspension was used for the peptone test.

Table XVI shows the result of the gelatin test.

TABLE XVI.

Stomach Extract on Gelatin.

15 cc. gelatin + HCl or NaOH + water + 0.5 cc. pepsin = 30 cc.

Tube No.	Added.		Initial pH.	Degree of liquefaction after digestion at 37° for			
	HCl	NaOH		15 min.	30 min.	60 min.	20 hrs.
1	10 cc. N	—	About 1.5	3	5	6	6
2	4 " "	—	2.5	5	6	6	6
3	8 " 0.1 N	—	3.0	4	6	6	6
4	3 " 0.1"	—	4.0	2	4	5	6
5	—	—	5.1	1	2	3	5
6	—	1.8 cc. 0.1 N	6.5	0	0	1	1
7	—	3.0 " 0.1"	7.3	0	0	0	0
8	—	4.0 " 0.1"	7.8	0	0	0	0
9	—	6.0 " 0.1"	8.5	0	0	0	0

It is obvious that the water extract from the stomach contains large amounts of pepsin. The results are in marked contrast to those obtained with water extracts from liver, pancreas, and spleen, which showed no trace of peptic enzymes. The optimum for the action is in the acid region and seems to lie about pH = 2 to 3. It is impossible to locate the optimum very accurately by this method, because in order to obtain hydrogen ion concentrations greater than pH = 2, such large amounts of acid must be added that they interfere with the liquefaction of the gelatin. The gelatin method as has been said is only useful for values of pH between 2 and 8.

Preceding authors have made accurate determinations of the optimum hydrogen ion concentration for pepsin, and found it to lie between a pH of 1 and 3. Sörensen (1909) found for the digestion of acid albumin an optimum pH of 1.5 which later was confirmed by Okada (1916). Recently Ringer (1916) has published a very interesting paper, showing that the optimum varies with the concentration and nature of the ions present. It is evident, however, from this experiment that there is no proteolytic action at all on gelatin above a pH of 6.

The question remains as to whether the mucous membrane of the stomach contains trypsin. It is not likely to be found in the

extract, because if it entered the acid solutions in the stomach it probably would be destroyed, as pancreatic trypsin is under similar conditions. For the peptone test, therefore, the mash has been used, the preparation of which is described above. Table XVII shows the results of this experiment.

TABLE XVII.

Stomach Cells on Peptone.

9 cc. peptone mixture + 1 cc. tissue suspension = 10 cc.
9 " " " contained 15.84 mg. total nitrogen.
1 " tissue suspension " 6.20 " " "

Tube No.	Initial pH.	Increase in the liberated nitrogen.
		mg.
1	5.0	<1.0
2	6.0	2.75
3	6.8	3.40
4	7.3	3.63
5	7.6	4.12
6	7.9	3.82
7	8.3	3.46

From this table it is quite evident that there are enzymes present which have the power of digesting Witte's peptone. The well marked optimum appears to lie at about pH = 7.6, about the same as for tryptic enzymes in the other tissues examined. That the pepsin itself cannot digest peptone in acid solution is shown in the following experiment.

2 cc. 4 per cent Witte's peptone solution + 4 cc. N HCl + 1 cc. pepsin solution + water = 10 cc.
At the beginning were obtained ..... 0.91 mg. N_2.
After 24 hours digestion at 37° ..... 0.90 " N_2.

These experiments have shown that there are in the ventricle, besides the well known pepsin, enzymes of he trypsin type, their presence however being largely obscured by the predominant action of pepsin.

Leukocytes.—Opie and later Dochez have studied the autolysis of polynuclear leukocytes and from their experiments concluded that proteolytic enzyme of leukocytes is active in alkaline solution. Parker and Franke (1917) claim to have demonstrated

ereptic enzymes in rabbit leukocytes, which attack peptones but not proteins in alkaline solution. Other authors who have studied this problem are Jobling, Eggstein, and Petersen (1915), Fiessinger and Clogne (1917), and Hiss (1908).

The leukocytes used in the following experiment were obtained by injecting sterile aleuronat into the pleural cavity of a rabbit. The exudate was centrifugalized, and the cells were carefully washed with salt solution. The deposit proved under the microscope to consist almost entirely of polynuclear cells, though a few mononuclear cells could be seen. About 0.5 gm. wet weight was obtained. To it was added chloroform, and it was allowed to stand for 24 hours at about 20° in order to plasmolyze. Then the cells were suspended in 25 cc. of distilled water, and this suspension was used as enzyme preparation. Table XVIII and Fig. 11 show the result of this experiment.

TABLE XVIII.

Leukocytes on Gelatin.

15 cc. gelatin + HCl or NaOH + water + 0.2 cc. leukocyte suspension = 30 cc.

Tube No.	Added.		Initial pH.	Degree of liquefaction after digestion at 37° for			
	HCl	NaOH		1 hr.	2 hrs.	6 hrs.	24 hrs.
1	6.0 cc. N	—	About 2.0	1	2	4	5
2	2.0 " "	—	2.7	2	4	6	6
3	10.0 " 0.1 N	—	3.2	2	4	5½	6
4	7.0 " 0.1"	—	3.7	1½	3½	5	6
5	9.0 " 0.1"	—	4.3	1	2	3	6
6	—	—	4.9	0	1.	2	4
7	—	1.2 cc. 0.1 N	6.0	0	0	1	2
8	—	1.8 " 0.1"	6.8	0	0	0	1
9	—	2.4 " 0.1"	7.3	0	0	1	1
10	—	3.2 " 0.1"	7.7	0	0	1	1
11	—	5.0 " 0.1"	8.3	0	0	1	1

The gelatin test indicates that leukocytes, like the other tissues examined, contain pepsin-like enzymes with maximum activity at pH = 3. There is no evidence of any proteolytic enzyme

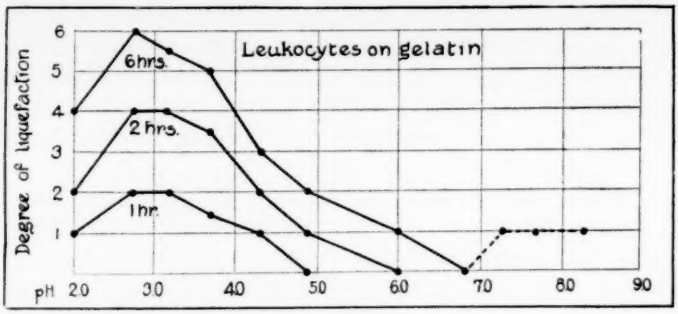

FIG. 11.

acting in alkaline solution on gelatin. Repeated experiments gave the same result.

The fact is noteworthy that the small quantity of leukocytes used could digest gelatin so rapidly. In the case of spleen or liver we had to use several hundred times as much tissue in order to get a corresponding action. The leukocytes evidently contain much greater amounts of pepsin-like enzymes than the other tissues. In this respect they resemble the yeast cells more than liver cells.

Table XIX shows the action of leukocytes on Witte's peptone.

TABLE XIX.

Leukocytes on Peptone.

9.0 cc. peptone mixture + 0.3 cc. leukocyte suspension + water = 10 cc.
Total nitrogen = 15.84 mg.
Amino " = 1.82 "

Tube No.	Initial pH.	Increase in the liberated amino nitrogen after 48 hrs. at 37°.
		mg.
1	4.0	0.17
2	5.3	0.17
3	6.8	0.25
4	7.3	0.85
5	7.6	1.02
6	7.9	1.68
7	8.5	0.25

Even if the resulting differences are small it is evident that there must also be trypsin-like enzymes present in the leukocytes, and their optimum reaction seems to be the same as that for the other animal tissues, between pH = 7.6 and pH = 8.0.

Autolysis.

The preceding experiments have yielded information concerning the nature of the proteolytic enzymes found in the tissues, and we have used a similar technique to study the nature of autolysis itself.

Liver.—Presumably because the glycogen present forms organic acids during autolysis, it is difficult to keep the hydrogen ion concentrations of liver mixtures constant during incubation. The following experiments show how strongly the hydrogen ion concentration increases.

Liver, autolyzed for 72 hours. No buffer present.

Tube No.	Initial pH.	pH after digestion.
1	6.1	5.5
2	7.3	6.4
3	7.9	6.8
4	8.4	6.9

Therefore we must use buffers and choose a rather short digestion time. In Table VI it is shown that the amino nitrogen formation at almost constant hydrogen ion concentration follows a curve of somewhat peculiar shape. We have two optimal points, one in the acid, the other in the alkaline region, and at about the neutral point there is almost no action at all. Table XX and Fig. 12 show a similar, more elaborate experiment.

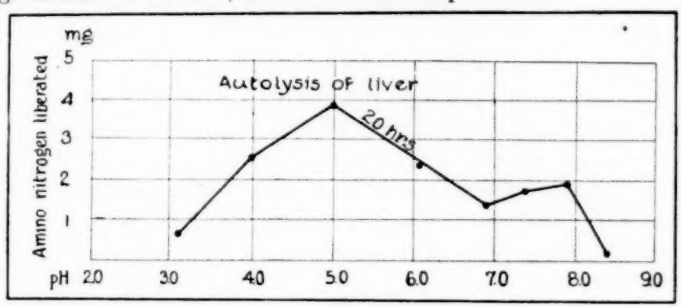

FIG. 12.

TABLE XX.

Autolysis of Liver.

1 cc. liver suspension (kept 3 days in ice box) + HCl or NaOH + 1 cc. 0.5 M phosphates + water = 10 cc.

Total nitrogen per 10 cc. = 22.60 mg.
Amino " " 10 " = 2.57 "

Tube No.	Added.				Initial pH.	After 20 hrs. digestion at 37°.		After 72 hrs. digestion at 37°.	
	0.1 N HCl.	0.1 N NaOH.	0.5 M phosphates.			Increase in amino N.	pH after digestion.	Increase in amino N.	pH after digestion.
			Na_2HPO_4	KH_2PO_4					
	cc.	*cc.*	*cc.*	*cc.*		*mg.*		*mg.*	
1	6.0	—	—	—	3.1	0.69	3.2	1.43	3.5
2	2.5	—	—	—	4.0	2.52	4.2	3.57	4.4
3	1.0	—	0	1.0	5.0	3.86	5.1	4.57	5.1
4	—	—	0.2	0.8	6.1	2.35	6.1	4.14	5.5
5		0.1	0.5	0.5	6.8	1.43	6.7	3.14	5.9
6		0.25	0.8	0.2	7.4	1.75	7.2	1.89	6.4
7		0.5	0.9	0.1	7.9	1.86	7.4	2.23	6.5
8		1.3	1.0	0	8.4	0.23	7.6	0.63	6.8

In spite of the phosphates present the hydrogen ion concentration increased appreciably, especially after 72 hours digestion. For this reason only the results after 20 hours digestion are used for plotting the curve. We note two well defined maxima in the curve, one at a pH of 7.8, the other at a pH of 5.5. There is greater action in the acid region, especially after longer duration of time. The optimum at pH = 7.8 is probably that of the tryptase which attacks the peptones already present. But what does the other maximum mean? Do we have another enzyme of trypsin type, acting in slightly acid solution? In such case we ought to have detected it before. Experiments in Tables IV to VI give no enzyme having the optimum pH = 5.5. But bearing in mind that the formation of amino nitrogen results from the action of both enzymes—the trypsin hydrolyzing the albumoses and peptones, first formed by the pepsin—it is easy to see that the optimum pH for the total action may well lie between the optima of the two individual enzymes and at a point where each exerts only a part of its maximum activity. The observed optimum at a pH of 5.5 occupies such a position.

Since pepsin produces only the more complex proteolytic products, such as albumoses and peptones, while trypsin produces

amino-acids and simple peptides, it is of interest to study the complexity of the products formed by autolysis at different hydrogen ion concentrations. When working with yeast it was found that in acid solutions considerable amounts of non-precipitable peptide nitrogen were present, whereas after autolysis in alkaline solutions practically all of the non-protein nitrogen was converted into amino nitrogen. A previous test gave the following result.

TABLE XXI.

Autolysis of Liver Products at Different Hydrogen Ion Concentrations.

25 cc. fresh pig liver suspension + HCl or NaOH + water = 100 cc.
Total nitrogen = 229.0 mg.
Amino " = 15.0 "

Tube No.	Added.		pH		Nitrogen in filtrate after 72 hrs. at 37°.			
	0.1 N HCl.	0.1 N NaOH.	Initial.	After digestion.	Total.	Protein.	Peptide.	Amino.
	cc.	cc.			mg.	mg.	mg.	mg.
1	20	—	4.2	4.0	177.0	9.0	63.0	64.5
2	—	10	7.6	7.0	132.0	24.0	0.0	51.8

As stated before it is impossible to keep the hydrogen ion concentration constant when working with liver, but when this organ is used it is determined before and after each digestion. Table XXII shows a similar experiment in which digestion was stopped

TABLE XXII.

Autolysis Products at Different Hydrogen Ion Concentrations.

25 cc. liver suspension (kept on ice for 7 days) + HCl or NaOH + water = 100 cc.

Total nitrogen = 402.0 mg.
Peptide " = 7.5 "
Amino " = 47.0 "
NH_3 " = 3.3 "

Tube No.	Added.	pH		Amount of non-precipitable nitrogen in filtrate after 48 hrs. at 37°.		
		Initial.	After digestion.	Peptide.	Amino.	NH_3.
				mg.	mg.	mg.
1	12.0 cc. N HCl.	2.8	3.1	33.2	57.3	3.3
2	3.0 " " HCl.	4.4	3.9	37.7	118.3	6.2
3	—	6.2	5.5	12.2	95.0	9.5
4	1.5 cc. N NaOH.	7.9	7.2	3.9	64.1	5.6

by adding trichloroacetic acid. The coagulum was removed and the amounts of peptide, amino, and ammonia nitrogen were determined as described under "General methods."

It is evident from the table that when autolysis has proceeded in acid solution a large proportion of peptide nitrogen still is left; that is, almost all of the dissolved proteins have been split to intermediate products, albumoses and peptones. But after autolysis in alkaline solution we have a large amount of intact soluble proteins and amino-acids, with no intermediate products.

Bearing in mind that the pepsin of the tissues is active only in acid, and the tryptase only in alkaline solution, the results described may readily be explained without any assumption of hypothetical activators or catalysts, or pro- or antienzymes.

Effect of Pre-Treatment with Acids.—Preceding authors have paid much attention to the phenomenon that a piece of tissue does not autolyze in alkaline solution, but if pre-treated with acid and then transferred into alkaline solution it will undergo a rapid autolysis. In the light of the results reported above the following explanation of the fact seems probable. In alkaline solutions the tissue pepsin does not attack most of the proteins, and the trypsin can digest only the peptones which are present from the beginning. But during the pre-treatment with acid the pepsin becomes active and the proteins are partially converted into peptones, which in the alkaline solution later proceed to undergo further digestion by the tissue trypsin. Without the pre-treatment in acid most of the material would have remained in the form of native protein indigestible by trypsin.

Here are given the results of an experiment which was carried out in the following manner. 3 equal parts of mashed liver were put into Erlenmeyer flasks, to two of them, a_1 and a_2, were added the same amounts of hydrochloric acid, and to the third, b, was added sodium hydroxide. The amount of amino nitrogen in them was determined at the beginning. After a certain time so much sodium hydroxide was added to a_2 that the hydrogen ion concentration became the same as in b. To the other two were added the same volume of water, and all were allowed to autolyze for the same time. Then the increase in amino nitrogen was determined.

TABLE XXIII.

Influence of Pre-Treatment with Acids.

a_1 and a_2: 5 cc. liver suspension (7 days in ice box) + 5 cc. 0.1 N HCl + water = 25 cc.

b: 5 cc. liver suspension + 18 cc. 0.1 N NaOH + water = 25 cc.

Total nitrogen = **4.30** mg.

Amino " = 4.70 "

	a_1		a_2		b	
	Increase in amino N.	pH	Increase in amino N.	pH	Increase in amino N.	pH
	mg.		*mg.*		*mg.*	
At the beginning	—	4.7	—	4.7	—	7.8
After 20 hrs. digestion at 24°	1.79	4.5	1.79	4.5, transferred to 7.8	1.34	7.5
" 24 " additional digestion at 37°	3.55	4.2	2.79	7.3	1.46	7.1

Spleen.—Spleen is a more favorable tissue for studies of autolysis than liver, because it does not contain so much glycogen, the decomposition of which raises the hydrogen ion concentration during the digestion.

Experiments detailed on previous pages have shown that the liberation of amino nitrogen by autolysis at constant hydrogen ion concentration follows the same scheme as for liver. Table XXIV and Fig. 13 show the results.

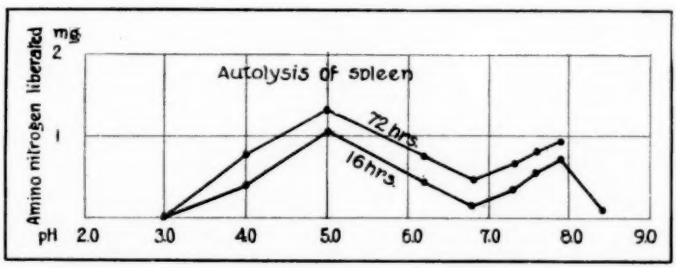

FIG. 13.

TABLE XXIV.

Autolysis of Spleen.

1 cc. spleen suspension (fresh) + HCl or NaOH + 1 cc. 0.5 M phosphates + water = 10 cc.

Total nitrogen = 6.8 mg.
Amino " = 0.53 "

Tube No.	Added.				Initial pH.*	Increase in amino-acid per 10 cc. at 37°.	
	0.1 N HCl.	0.1 N NaOH.	Phosphates.			After 16 hrs.	After 72 hrs.
			Na_2HPO_4	KH_2PO_4			
	cc.	cc.	cc.	cc.		mg.	mg.
1	6.0	—	—	—	About 3.0	0.03	0.06
2	2.5	—	—	—	" 4.0	0.40	0.78
3	1.0	—	0	1.0	5.0	1.06	1.28
4	—	—	0.2	0.8	6.2	0.43	0.73
5	—	—	0.5	0.5	6.8	0.15	0.43
6	—	0.1	0.8	0.2	7.3	0.38	0.69
7	—	0.2	0.9	0.1	7.6	0.55	0.78
8	—	0.4	1.0	0	7.9	0.74	0.98
9	—	1.0	1.0	0	8.4	0.09	—

* In this case the hydrogen ion concentration did not change so much as in the case of liver. On the acid side the hydrogen ion concentration remained rather constant; on the alkaline, however, it decreased 0.2 to 0.3 in pH during the digestion.

TABLE XXV.

Autolysis of Spleen and Products at Different Hydrogen Ion Concentrations.

25 cc. spleen suspension (quite fresh) + HCl or NaOH + water = 100 cc.

100 cc. contained 327.5 mg. total nitrogen.
100 " " 17.7 " peptide "
100 " " 25.5 " amino "
100 " " 2.3 " NH_3 "

Tube No.	Added.	pH		Amount of non-precipitable nitrogen after 48 hrs. at 37°.		
		Initial.	After digestion.	Peptide.	Amino.	NH_3.
				mg.	mg.	mg.
1	9.0 cc. 0.1 N HCl.	2.9	3.1	24.8	38.5	2.3
2	1.0 " 0.1 " HCl.	4.2	3.9	55.2	142.3	6.2
3	—	6.3	5.9	2.6	87.9	7.3
4	1.0 cc. 0.1 N NaOH.	7.6	7.3	3.3	83.0	5.1

We have here two optima, one at a pH of about 8, the other at a pH of about 5. The explanation is the same as for the corresponding experiment with liver.

Table XXV shows the autolysis products at different hydrogen ion concentrations. It is analogous to Table XXII.

The most interesting results are to be found under "Peptide nitrogen." When autolysis has taken place in acid solutions, there are considerable amounts of intact peptides, whereas in alkaline

TABLE XXVI.

Influence of Pre-Treatment with Acids.

a_1 and a_2: 5 cc. spleen suspension (6 days in ice box) + 4.0 cc. 0.1 N HCl + water = 20 cc.
b: 5 cc. spleen suspension + 1.0 cc. 0.1 N NaOH + water = 20 cc.
a_2 neutralized after 16 hrs. with 5.5 cc. 0.1 N NaOH.
Total nitrogen = 34.0 mg.
Amino " = 2.68 "

	a_1		a_2		b	
	Increase in amino N.	pH	Increase in amino N.	pH	Increase in amino N.	pH
	mg.		*mg.*		*mg.*	
At the beginning	—	4.0	—	4.0	—	7.8
After 16 hrs. digestion at 24°	5.05	4.0	5.05	4.0, transferred to 7.8	1.20	7.8
" 18 " " " 37°	7.25	4.0	7.91	7.6	3.52	7.6
" 72 " " " 37°	8.10	4.2	9.36	7.6	3.94	7.6

solutions almost all peptides are split into amino-acids. This is the same result as obtained with liver, and subject to the same explanation.

Tables XXVI and XXVII show the influence of pre-treatment with acids and subsequent transfer to alkaline solution. In both cases autolysis proceeds much more rapidly in the transferred sample than in the one where the reaction is alkaline from the beginning. Here also the results are the same as with liver.

Pancreas.—Most authors have found that pancreas tissue autolyzes much more rapidly than liver and spleen. This is un-

TABLE XXVII.

a_1 and a_2: 5 cc. spleen suspension (fresh) + 2 cc. 0.1 N HCl + water = 20 cc.

b: 5 cc. spleen suspension + 0.8 cc. 0.1 N NaOH + water = 20 cc.

a_2 neutralized after 6 hrs. at 37° with 2.8 cc. 0.1 N NaOH.

Total nitrogen = 16.0 mg.

Amino " = 1.52 "

	a_1		a_2		b	
	Increase in amino N.	pH	Increase in amino N.	pH	Increase in amino N.	pH
	mg.		*mg.*		*mg.*	
At the beginning	—	4.7	—	4.7	—	7.6
After 6 hrs. digestion at 37°	0.31	4.7	0.31	4.7, transferred to 7.6	0.12 0.65	7.6 7.5
" 24 " " " 37°	2.58	4.6	1.80	7.6		

TABLE XXVIII.

Autolysis of Pancreas.

1 cc. pancreas suspension (fresh) + HCl or NaOH + 1 cc. 0.5 M phosphates + water = 10 cc.

10 cc. contained 17.4 mg. total nitrogen.

10 " " 1.26 " amino "

Tube No.	Added.				Initial pH.	Increase in amino-acids per 10 cc. after digestion at 37° for	
	0.1 N HCl.	0.1 N NaOH.	Phosphates.			20 hrs.	72 hrs.
			Na_2HPO_4	KH_2PO_4			
	cc.	*cc.*	*cc.*	*cc.*		*mg.*	*mg.*
1	6.0	—	—	—	About 3.0	—	1.37
2	2.5	—	—	—	" 4.0	0.23	3.17
3	1.0	—	0	1.0	5.0	2.40	5 12
4	—	—	0.2	0.8	6.2	3.59	6.08
5	—	—	0.5	0.5	6.8	4.00	6.70
6	—	0.1	0.8	0.2	7.3	4.27	7.12
7	—	0.2	0.9	0.1	7.6	4.12	—
8	—	0.5	1.0	0	7.9	4.00	7.19
9	—	1.2	1.0	0	8.4	1.31	3.27

doubtedly due to the great amounts of free trypsin present. Yet the pancreas is not a good subject for studying the general problem of autolysis because the action of the trypsin almost entirely obscures the actions of the other proteolytic enzymes. This has been shown in Table XI.

Table XXVIII is quite similar to Tables XX and XXIV, and shows the liberation of amino nitrogen during autolysis at constant hydrogen ion concentration. Fig. 14 represents the results graphically.

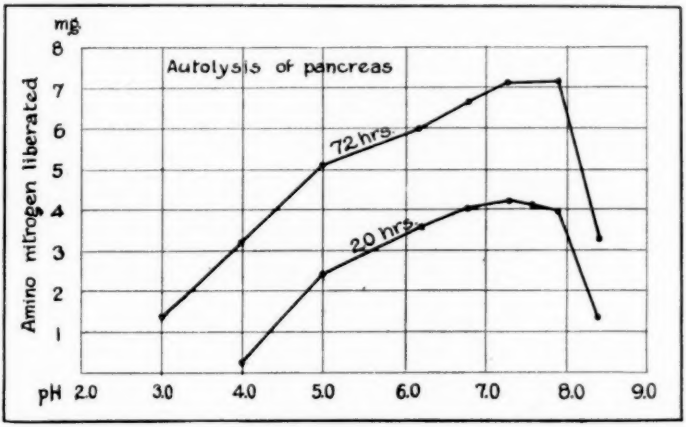

FIG. 14.

In the case of the liver and spleen we obtained two maximum points, one in the acid and one in the alkaline region. But here we only obtain one maximum point, at about pH = 7.8, which also is the maximum for trypsin. Even in the acid region, however, there is a considerable proteolytic activity. Whether this is due to the trypsin or to the pepsin or to both is impossible to state.

Because of this irregular behavior of the pancreas it will hardly be of value to find out the autolytic products at different hydrogen ion concentrations. Table XXIX shows the influence of pretreatment with acids on the subsequent autolysis in alkaline solution. It is quite similar to the corresponding experiments on liver and spleen, and gives the same result.

TABLE XXIX.

Influence of Pre-Treatment with Acids.

a_1 and a_2: 5 cc. pancreas suspension (7 days in ice box) + 3 cc. 0.1 N HCl + water = 20 cc.

b: 5 cc. pancreas suspension + 1 cc. 0.1 N NaOH + water = 20 cc.

After 6 hrs. digestion at 37° a_2 neutralized with 3.6 cc 0.1 N NaOH.

Total nitrogen in 20 cc. = 63.1 mg.

Amino " " 20 " = 10.2 "

	a_1		a_2		b	
	Increase in amino N.	pH	Increase in amino N.	pH	Increase in amino N.	pH
	mg.		*mg.*		*mg.*	
At the beginning	—	4.8	—	4.8	—	7.8
After 6 hrs. digestion at 37°.	2.70	4.9	2.70	4.9, transferred to 7.8	1.75	7.8
After subsequent 24 hrs. digestion at 37°	9.60	5.0	10.80	7.7	7.80	7.8

Influence of Neutral Salts on Animal Enzymes.

The erepsin of the intestines behaves in regard to neutral salts quite differently from ereptase of yeast. The ereptic action is markedly checked already in 0.1 normal salt solution, while the yeast ereptase is not influenced in less than about normal (Dernby, 1917). Whether this sensitiveness to salt concentration is shared by the other animal enzymes remains to be determined. It is likely that most of the enzymes from animal tissue are as sensitive to salts as erepsin.

SUMMARY.

In this paper the proteolytic enzymes of liver, spleen, pancreas, and leukocytes, and of the mucous membrane of the stomach have been studied. In all of these tissues it has been possible to demonstrate the existence of pepsin-like enzymes, which split native proteins to peptones but not further, and for whose action the optimum hydrogen ion concentration in all cases is at about pH = 3.5. This enzyme cannot act in alkaline or even neutral solutions.

It has also been possible to show that in all of these tissues there are proteolytic enzymes of the type of trypsin or erepsin that attack only peptones or peptides, and split them into amino-acids. The optimum hydrogen ion concentration for all these enzymes seems to be about pH = 7.8 (from 7.6 to 8.0), and their action is checked in slightly acid solution.

Both types of proteolytic enzymes occur in all the tissues studied. In some of them, however, one or the other of the enzymes has been predominant over the others, as the pepsin in the stomach and the trypsin in the pancreas. Unlike the less specialized enzymes, pancreatic trypsin and gastric pepsin can easily be extracted from the cells.

Autolysis of pancreas and liver has been especially studied in regard to the hydrogen ion concentration of the solution. It has

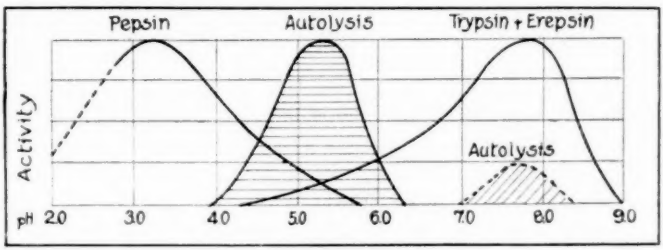

FIG. 15.

been found that self-decomposition of the tissues proceeds furthest in a range of pH between 5 and 6. That is in slightly acid solution and the optimum lies between those of pepsin and trypsin. The explanation is that the autolysis is most rapid and complete when both types of enzymes can work simultaneously.

The relations between hydrogen ion concentration and the different proteolytic enzymes as well as the autolysis of animal tissues are represented in Fig. 15.

When a piece of tissue is first treated with weak acids and subsequently transferred into alkaline solution the autolysis here proceeds much farther than if the tissue has been from the start in alkaline solution. This fact, shown previously by Hedin and by Dochez, is explained as follows. During the preliminary treatment in acid solution the pepsin acts. By transfer to alka-

line solution its action is checked, and the trypsin works under optimum conditions and digests the peptones formed by the action of the pepsin.

Considering that there are enzymes in the tissues of both peptic and tryptic nature and considering also the hydrogen ion concentration of the medium and its eventual changes, most of the autolytic reactions can be explained without assumption of hypothetical agents, such as "proenzymes," "activators" or "antienzymes."

Comparing the proteolytic actions in the yeast cells with those of animal tissues, the following is obtained:

Proteolytic Enzymes.	*Optimum Hydrogen Ion Concentration for the Enzymes.*	
	Yeast cell. *pH*	Animal tissues. *pH*
Pepsin	4.0–4.5	3.0–3.5
Trypsin	About 7	7.8
Erepsin	7.8	7.8

In what sense the hydrogen ion concentration will change during autolysis is impossible to predict. This depends upon the chemical constituents of different tissues and might vary in any direction. When starting at about pH = 6 in buffer-free solution with a glycogen-free tissue the most common result is that the hydrogen ion concentration first increases, reaches a maximum, and then decreases considerably.

This work has been carried out in the laboratory of Dr. Donald D. Van Slyke, and I wish to express my great indebtedness to him for his interest in this work. I am also indebted to Dr. J. Auer for supplying me with the animal tissues.

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THE PURINE METABOLISM OF THE DALMATIAN COACH HOUND.

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Recently Benedict[1] reported the observation that the Dalmatian breed of coach hound exhibits a marked peculiarity in its purine metabolism, to the extent that it excretes large amounts of uric acid, even when on a purine-free diet. This peculiarity was exhibited by four Dalmatians examined, but a fifth, "obviously not of very pure breed," did not eliminate much uric acid. That a single species of dog should exhibit this characteristic is remarkable, especially in view of the fact that the only other mammals that excrete uric acid in any considerable amounts are man and the anthropoid apes. The Dalmatian coach hound, however, differs also from man and the anthropoids, in that it excretes allantoin in considerable amounts; *i.e.*, from one-half to two-thirds as much allantoin as uric acid. Hence the Dalmatian is unique in respect to purine metabolism.

This fact is of more than academic interest, for it gives us for the first time a laboratory mammal that excretes considerable amounts of uric acid, and hence serves as a possible medium for experimentation under conditions somewhat resembling those obtaining in the human subject. Benedict has already employed the Dalmatian for such investigations. Since other species of dogs differ so radically from the Dalmatian in respect to purine metabolism, there is also opened a most interesting field of investigation in the inheritance of a metabolic process, as exhibited by the products of mating of Dalmatians with other species.

Having found an apparently pure blooded Dalmatian in one of our laboratories it seemed desirable to determine whether it was

[1] Benedict, S. R., *J. Lab. and Clin. Med.*, 1916–17, ii, 1.

equipped with the same outfit of purine enzymes as other dogs, or not. We first made several observations at different times on the uric acid excretion, and corroborated Benedict's findings completely. Uric acid was determined by direct isolation and weighing of the crystalline product, in order to avoid the possibility of any unknown substance being responsible for the results obtained. The animal was a female, the weight being about 13.5 kilos, and it was given a liberal diet, chiefly meat. On this diet the excretion of uric acid was found to be as follows:

	Urine.	Uric Acid.
	cc.	*gm.*
Apr. 25	573	0.3673
May 10	610	0.5132
" 13	740	0.6320
June 15	351	0.4409
July 5	910	0.5425
Average daily excretion		0.499

This rate of excretion, 0.037 gm. per kilo, corresponds to a daily excretion of 2.59 gm. of uric acid by a 70 kilo man.

Benedict found about 0.360 gm. of uric acid excretion daily by his 10 kilo dog, or 0.036 gm. per kilo, even on a purine-free diet. When thymus was fed the uric acid rose to as high as 0.600 gm. of uric acid per day, but at the same time the allantoin nitrogen rose from 0.050 to 0.210 gm. Evidently this dog was able to destroy a large part of the exogenous purines, but not all. When uric acid was injected subcutaneously it was recovered quantitatively in the urine, yet there was also a marked increase in the allantoin excretion. These findings are difficult to explain on the basis of our present knowledge of purine metabolism, and Benedict is obliged to suggest that, "It seems probable that uric acid and allantoin are interrelated in metabolism in other ways than have been heretofore assumed."[2]

[2] This article was written in June, 1917, but its publication was delayed during the author's absence in foreign service. In October, 1917, C. K. Watanabe published a paper (*J. Urol.*, 1917, i. 485) on experimental uranium nephritis in two Dalmatian coach hounds, in which animals he observed excretion of uric acid, but in much smaller amounts than found by Benedict or with my dog. Dr. M. H. Givens at Yale has analyzed the urine of two fasting Dalmatians, obtaining figures more

In studying the purine enzymes of this dog the same methods were used as described in previous papers from this laboratory.[3] The dog was killed with chloroform, and the tissues, ground to a pulp, were allowed to act upon uric acid and purines both aerobically and anaerobically, with the following results.

Experiment 1.—80 gm. of liver pulp were added to 0.240 gm. of uric acid dissolved with a minimum amount of NaOH in boiling water. The mixture was digested with toluene at 38–40°C. for 24 hours, with a constant stream of warm moist air bubbling through it. At the end of this time the proteins were removed by coagulation and filtration. The filtrate contained only a very small amount of purine material, in which not a trace of uric acid could be detected. There was recovered from the scanty purine precipitate about 0.045 gm. of xanthine. *The liver of this dog contains uricase.*

Experiment 2.—80 gm. of liver pulp were digested with 0.229 gm. of xanthine under the same aerobic conditions as described above. After the digestion there could be detected no uric acid in the mixture, but a considerable amount of xanthine, 0.150 gm. being recovered. This experiment indicates that the liver of this dog exhibits less xanthine-oxidase activity than is seen in other species of dogs, for ordinary dog liver tissue would have converted the xanthine into uric acid and destroyed it quantitatively under these conditions. The rather low recovery suggests that possibly there has been some xanthine-oxidase activity, but does not establish it, since there are sources of quantitative error in this work.

closely resembling those of Benedict and myself. He has authorized me to publish these results, which were as follows:

Weight of dog. *kg.*	Volume. *cc.*	Uric Acid. N. *mg.*
16	455	214
	190	208
	240	208
10.5	330	232
	335	233
	350	213

These figures show a daily excretion of 0.640 gm. and 0.678 gm. of uric acid for these dogs, or respectively 0.040 gm. and 0.064 gm. per kilo.

[3] Wells, H. G., *J. Biol. Chem.*, 1909–10, vii, 171. Wells, H. G., and Caldwell, G. T., *ibid.*, 1914, xviii, 157.

The recovery of a small amount of xanthine in Experiment 1, furthermore, implies an almost or quite complete lack of xanthine oxidase.

Experiment 3.—Control to Experiment 1. 80 gm. of liver pulp were added to 0.240 gm. of uric acid, in solution, and the mixture was immediately brought to 100°C., and held at this temperature for 30 minutes. 0.205 gm. of uric acid was recovered, after incubating with an air current as in Experiment 1.

Experiment 4.—62 gm. of kidney pulp were added to 0.240 gm. of uric acid and digested aerobically. 0.150 gm. of uric acid was recovered. Possibly there was a slight uricolytic activity by the kidney, but repetition of this result would be necessary to prove it.

Experiment 5.—31 gm. of spleen pulp were added to 0.229 gm. of xanthine and digested aerobically. No uric acid could be recovered or detected but 0.155 gm. of xanthine was found. Evidently the spleen lacks xanthine oxidase.

Experiment 6.—80 gm. of liver pulp were placed in 400 cc. of water containing 0.120 gm. of guanine and 0.154 gm. of adenine. The mixture was digested under toluene, without aeration, for 7 days at 37°. Analysis yielded no guanine, 0.208 gm. of xanthine, no adenine, and 0.172 gm. of hypoxanthine. Evidently the liver possesses guanase, and converts added guanine into xanthine; also apparently it converts some of its own purines into xanthine. It also contains adenase which converts adenine into hypoxanthine.

From these experiments on a single specimen of Dalmatian coach hound, it seems that this animal had uricase in its liver, but probably not in the kidney. This observation indicates that the constant excretion of large amounts of uric acid does not depend upon the absence of uricase from the tissues of this breed of dog, although possibly there is a quantitative deficiency in uricase activity that is not disclosed by our experiments. Further studies are necessary to determine this, but as yet no more Dalmatians have become available. A surprising result is the apparent absence of xanthine oxidase in both the liver and spleen. It is unfortunate that we were unable to examine all the tissues of this dog to learn where it forms its uric acid. The absence of uricase from the kidney is to be expected in view of the free passage

of uric acid through this organ. The presence of adenase and guanase in the liver indicates that in respect to the possession of these two enzymes the Dalmatian does not differ from other dogs, although we do not know whether these enzymes have the same wider distribution as in other species of dogs.

SUMMARY.

In corroboration of Benedict's observation, a Dalmatian coach hound was found to excrete large quantities of uric acid. The liver of this same dog possessed the power of destroying uric acid *in vitro*, indicating that the presence of uric acid in the urine of the Dalmatian does not depend on the absence of uricase in its tissues. The kidney did not exhibit uricolytic activity. Neither the liver nor spleen converted xanthine into uric acid, but the liver deaminized both guanine and adenine.

A NEW FORM OF COLORIMETER.

By JOSEPH C. BOCK and STANLEY R. BENEDICT.

(From the Department of Chemistry, Cornell University Medical College, New York City.)

Plates 1 to 3.

(Received for publication, June 19, 1918.)

Recent years have witnessed a phenomenal growth in the number of colorimetric methods applicable in biological chemistry; hence the colorimeter has become one of the instruments of prime importance to the biological chemist. Indeed, it is probably not an exaggeration to regard one or more colorimeters as part of the necessary equipment of the laboratory worker, the scientific clinician, and the medical student.

The Duboscq colorimeter, introduced to the biological chemist by Folin, has until recently met practically all the demands for an ideal colorimeter. The expense of this instrument, together with the difficulty of obtaining it at present, has rendered it imperative that another colorimeter be developed in this country which should be both accurate and reasonable in price. These conditions have not, in our opinion, been met by any of the Duboscq type of colorimeters manufactured in this country at the present time.

In devising the colorimeter to be described in the present communication we have endeavored to construct an instrument which should meet all the requirements of accurate colorimetry, as employed in biological chemistry, but which should be as simple in construction as is consistent with meeting these requirements.

In our instrument the costly and difficultly obtainable prisms employed in the Duboscq and similar instruments have been replaced by mirrors.[1] A common objection to mirrors in a

[1] Colorimeters using mirrors have been described by several writers. In the arrangement of mirrors the well known Campbell-Hurley instrument most closely approximates our own.

colorimeter has been that their use involves too great a loss of light. We have found that where there is only a single reflection of the light and where very thin mirrors are used the loss of light is negligible. First-surface mirrors would be theoretically the best, but these are so easily ruined by necessary handling or cleaning that they cannot be used. We have, therefore, employed mirrors closely approximating first-surface mirrors, obtained by silvering the ordinary microscope cover-slip. The arrangement of the mirrors will be made plain by reference to Figs. 1, 2, and 3.

The standard solution employed in colorimetry is always set at a fixed height. We have, therefore, deemed it unnecessary to have a movable plunger for the standard solution but have substituted for this the use of a cell of known dimension, such as 15 × 20 mm. This makes it possible to simplify the construction of the instrument greatly without impairing its value, since cells may be used from 5 to 20 mm. in diameter.

Since colorimetric readings in biological chemistry seldom require a greater depth than 25 mm., we have arranged for the movable cup to read only from zero to a depth of about 30 mm. This, again, is done to cut the cost of manufacture and does not, we believe, impair the usefulness of the instrument. If necessary, a dilution of the standard will compensate for the shorter range of the scale.

Fig. 1 shows the colorimeter with the housing taken off and the diaphragm and standard cell removed.[2] The illuminating mirror M is made by plating finely ground glass. This kind of mirror is used to prevent direct reflection of objects being visible in the reflecting mirrors A and B. The illuminating mirror throws the light onto the reflecting mirrors A and B. The mirror B is so arranged as to take up half of the field of vision in the small telescope T. B is placed over the plunger, giving a reflection of color in the unknown solution which is contained in the movable cup C. Between A and B a cell K containing the standard solution is placed. This cell is made of optical glass and the inside dimensions are exactly known. The light from M is reflected by A and goes through the cell K and reaches the telescope making

[2] We are much indebted to Mr. G. F. Soderstrom, of the Department of Physiology, Cornell University Medical College, for many helpful suggestions concerning the mechanical make-up of the instrument.

up the right side of the field. This side of the field is not changeable, whereas the left half, picturing the liquid column under the plunger, changes with the up and down movement of the cups containing the unknown solution. The standard is contained in the cell K.

It is planned at present to have two cells for each instrument, one having inside dimensions of 5 × 10 mm. and the other 15 × 20 mm. When filled to the top, the cup holds only about 5 cc. so that when the scale reading is about 30 mm. the cup contains approximately 2 cc. of liquid, allowing work with very small quantities.

The rack and pinion are of brass and steel respectively, this combination being supposed to be the best safeguard against wear. All parts of the instrument are easily renewed in case they are broken; even the reflecting mirrors are replaceable in a few minutes. The plunger is adjustable and allows corrections in case the replacing of a cup or plunger should make this necessary. The telescope contains only a single lens. A small diaphragm D (Fig. 2), is put between the mirrors and the telescope to yield a sharp and perfectly focused field. The top piece of the instrument, containing the mirrors and cell, is protected by a removable housing H (Fig. 3). The whole instrument is very light and, at the same time, well balanced. A large spreading base makes the accidental upsetting of the apparatus very difficult.

In order to use the colorimeter put empty, dry cell in place, put on housing, and the cup at a distance so as to be equal to cell diameter. Looking through the telescope, move the illuminating mirror until both fields are exactly equal. Now put standard solution in the cell and unknown solution in the cup. Move cup slowly up and down by means of pinion wheel until fields appear even. Read scale. By turning the cell 90° another depth of standard color is obtained. All readings must be taken with housing in place (Fig. 3).

The instrument in its present form is adapted to nearly all colorimetric work. We expect later to adapt the instrument to nephelometric work and are making investigations along this line at present.

Concerning results obtainable with the new colorimeter, we may state that the readings appear quite as sharp as those given by

the best Duboscq instrument. We did not expect originally that a mirror instrument could yield the same light field and the sharp color change of the Duboscq instrument but 6 months of continuous work with the new instrument have convinced us that readings with this are as rapidly and as accurately made as with the Duboscq colorimeter.

The new instrument is being made by the Klett Manufacturing Company, Inc., 202 East 46th Street, New York, and will cost about $35.

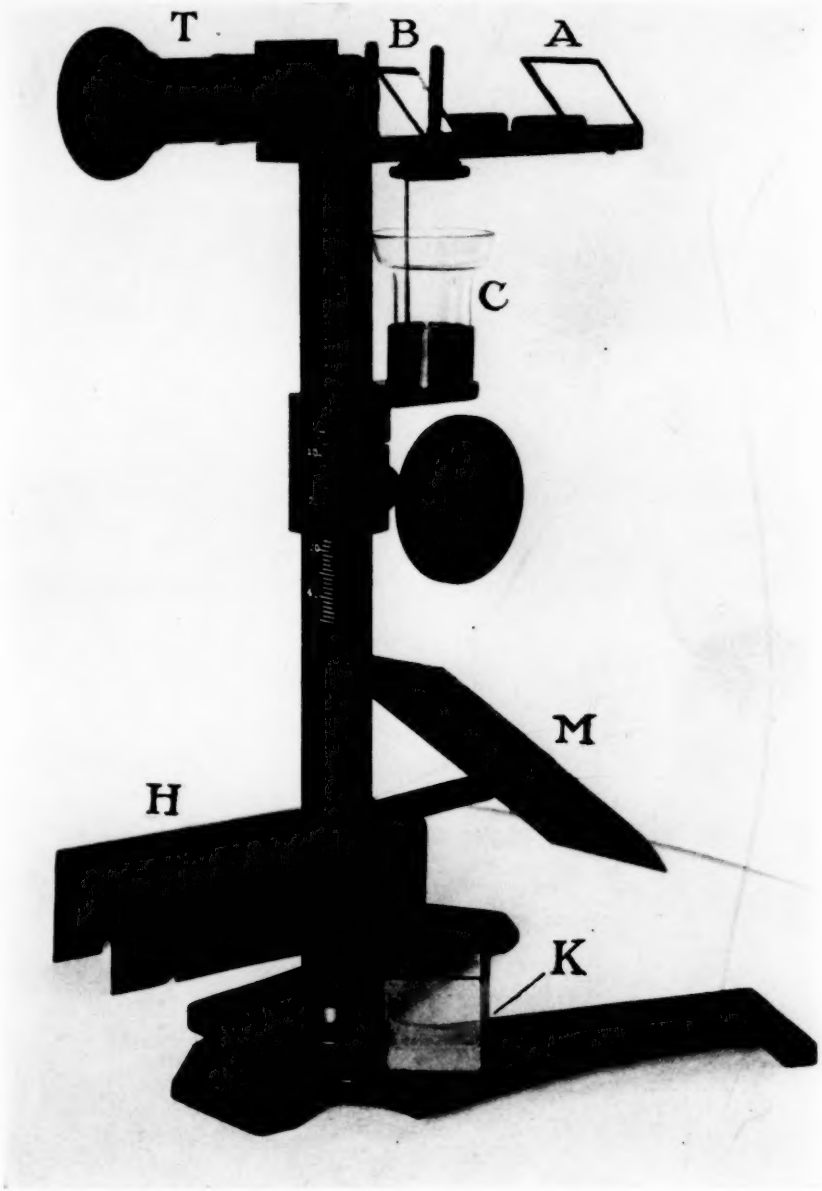

FIG. 1.

(Bock and Benedict: Colorimeter.)

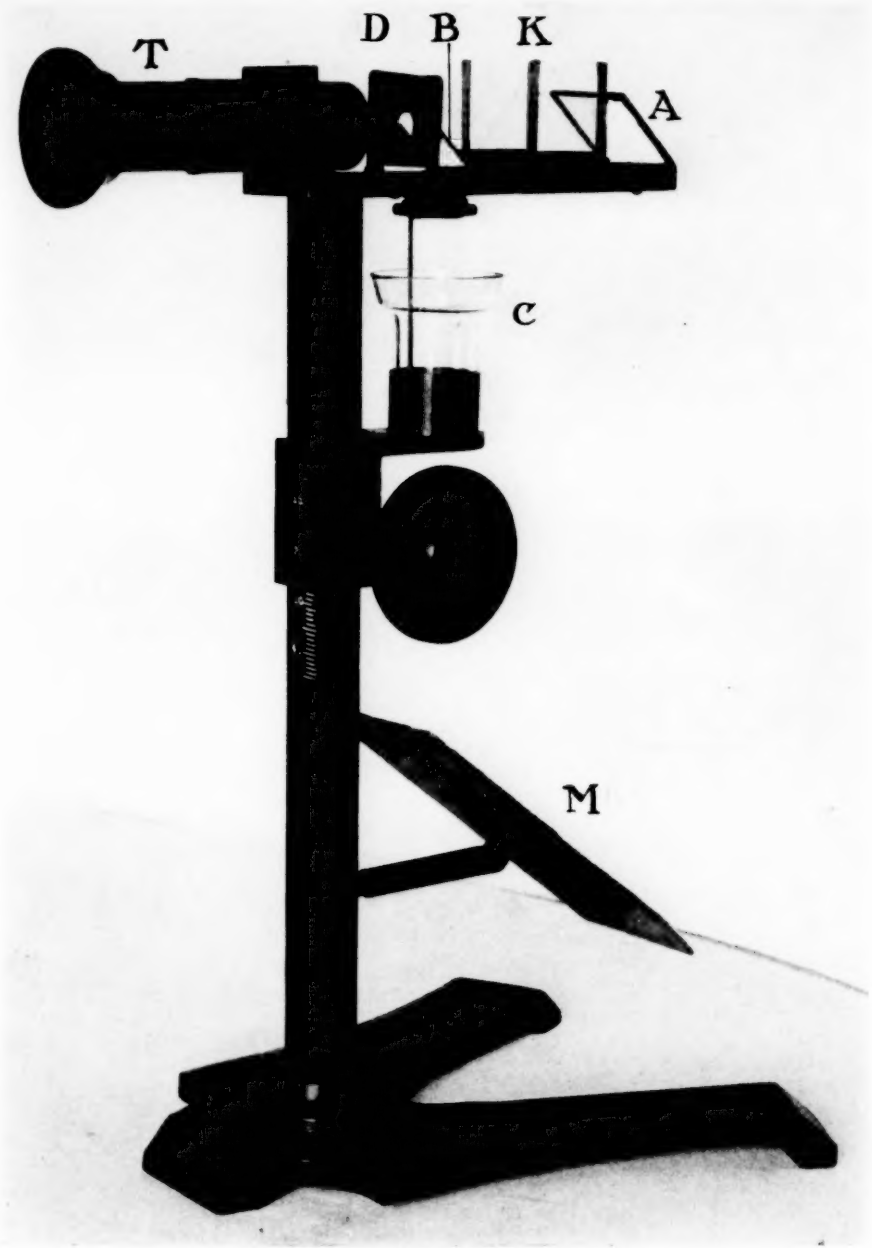

FIG. 2.

(Bock and Benedict: Colorimeter.)

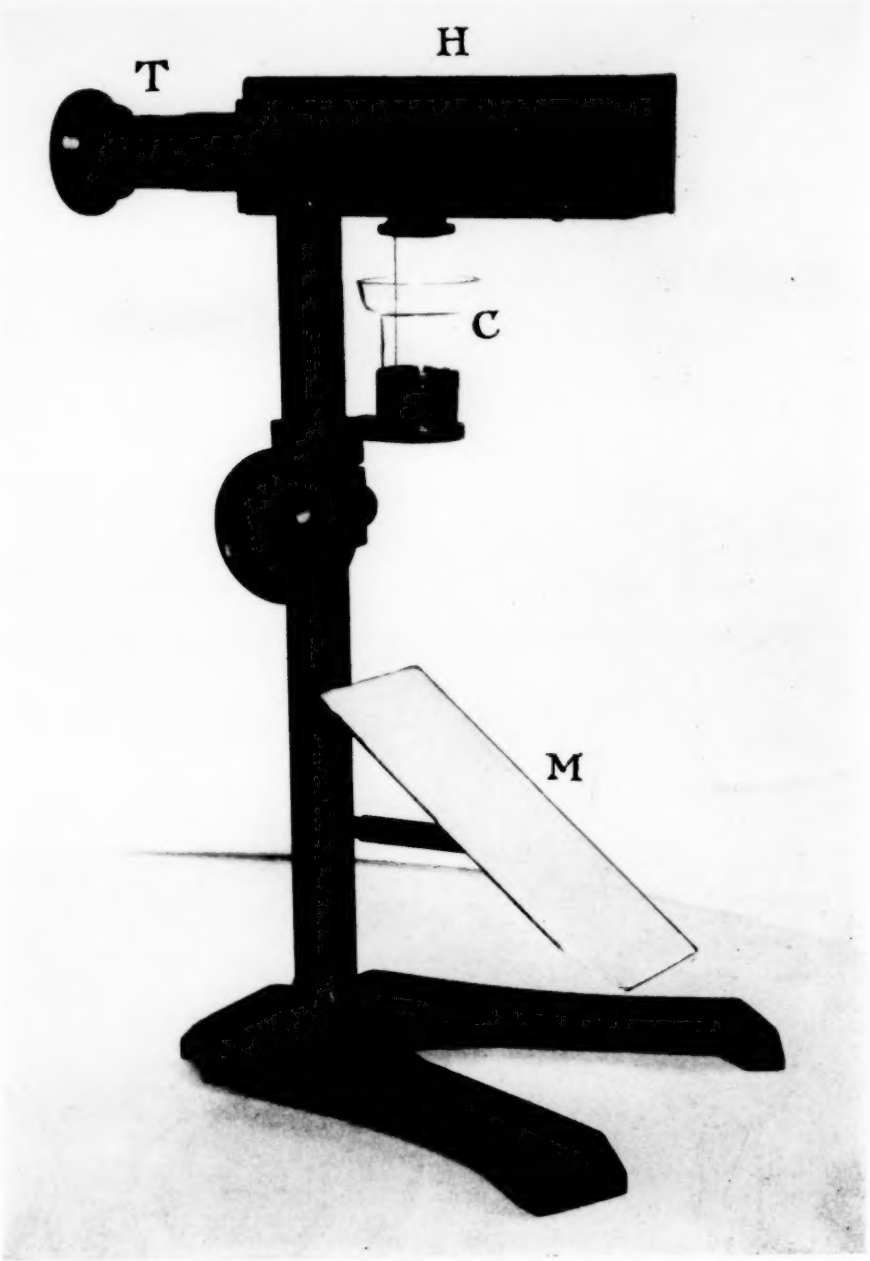

FIG. 3.

(Bock and Benedict: Colorimeter.)

A RAPID METHOD FOR THE DETERMINATION OF IRON IN SMALL QUANTITIES OF BLOOD.

By LOUIS BERMAN.

(*From the Chemical Laboratory of the Berwind Maternity Clinic, New York.*)

(Received for publication, May 21, 1918.)

In the method to be described the iron held in combination in fluid blood is split off by the action of concentrated hydrobromic acid. The iron is oxidized to the ferric condition and the organic matter is destroyed by potassium permanganate. The resultant solution is mixed with a solution of ammonium sulfocyanate in water and acetone and the color is compared with that of a standard iron solution similarly treated.

The first to use the sulfocyanate method for the determination of iron in blood colorimetrically was apparently Lapicque.[1] He ashed 2 gm. of blood with H_2SO_4. As his normal figure he obtained 44 mg. of Fe per 100 parts of blood. Some years after, Jolles[2] published his clinical ferrometer. He, too, ashed a fairly large quantity of blood, but compared his colors in the Fleischl-Miescher hemoglobinometer. As his normal figure he established 62 mg. per 100 cc. His method was severely criticized by J. Hladik[3] on various grounds. Jolles, however, has continued his claims, and has published a number of articles.[4] Recently,[5] he reviewed his method, still claiming accuracy for it. However, it has been discredited by a number of workers, among them Rosin and Jellinek[6] and Mayer.[7] There have been a number of other attempts to work out an accurate method of applying the sulfocyanate reaction to blood. Wolf and Marriott[8] introduced the use of acetone to improve the color.

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[1] Lapicque, L., *Compt. rend. Soc. biol.*, 1889, xli, 167.

[2] Jolles, A., *Monatsh. Chem.*, 1896, xvii, 677.

[3] Hladik, J., *Wien. klin. Woch.*, 1898, xi, 74.

[4] Jolles, *Deutsch. med. Woch.*, 1897, xxiii, 148; 1898, xxiv, 104; *Wien. klin. Woch.*, 1899, xii, 672; *Centralbl. inn. Med.*, 1899, xx, 681.

[5] Jolles, *Centralbl. inn. Med.*, 1916, xxxvii, 1.

[6] Rosin, H., and Jellinek, S., *Z. klin. Med.*, 1900, xxxix, 109.

[7] Mayer, A., *Z. klin. Med.*, 1903, xlix, 475.

[8] Wolf, C. G. L., and Marriott, W. McK., *J. Biol. Chem.*, 1905–1906, i, 456.

A critical discussion of these various methods is beyond the scope of this paper. As was pointed out by Letsche[9] in all the methods in use, the blood is first ashed, and then a determination of the inorganic iron is made. This has demanded, for accurate work, the employment of large amounts of blood, as the degree of error produced by the manipulations in ashing and redissolving made it necessary. Besides, even with small amounts, the processes are long and tedious. In that of Wolf and Marriott, for example, an hour to an hour and a half are necessary even in practised hands. The time and number of steps involved explain why these methods never came into general use.

In the method to be described, the ashing process is eliminated, and a complete determination may be made in 10 to 15 minutes.

Method.

Blood is obtained in the usual manner for determining hemoglobin with the Sahli apparatus, by pricking the finger or ear lobe. To 0.040 cc. of blood, measured in a calibrated pipette, mixed with 2 cc. of water and 0.2 cc. of 0.1 N HCl, 2 cc. of 0.1 N $KMnO_4$ are added. Then the mixture is placed in a water bath for about 2 minutes, when a brownish red coagulum results, supernatant above a colorless or slightly yellowish fluid. To this two drops of concentrated hydrobromic acid are added. The tube is returned to the water bath for another 2 minutes. The product should be a water-clear fluid containing a few whitish flakes. The solution is filtered through acid-washed filter paper into a long narrow graduate. Washings are added until the filtrate has reached the 5 cc. mark. Then 5 N NH_4CNS is added up to 10 cc., and acetone up to 20. The graduate is stoppered, and the fluids are mixed. A shrinkage of volume occurs. Acetone is added again to the 20 cc. mark, and the graduate is stoppered. A salmon-red color is produced.

The standard, which may be prepared at the same time as the blood, is made by adding two drops of concentrated hydrobromic acid to 2 cc. of the standard iron solution containing 0.009 mg. of Fe per cc., and keeping it in the water bath for 2 to 4 minutes. It also is made up to 5 cc. with water, to 10 cc. with 5 N NH_4CNS, and to 20 cc. with acetone in a narrow graduate, which is stoppered

[9] Letsche, E., Handb. Biochem. Method, Berlin and Vienna, 1911, v, pt. i, 139.

and shaken. After the shrinkage incidental to the mixture of fluids, acetone is added up to 20 cc. and the graduate is stoppered and shaken.

Allow both graduates to stand for 5 minutes.

Comparison may be best made in a Duboscq or Kober colorimeter, with the standard set at 10. The calculation is $\frac{10}{R} \times 45$ = number of mg. of Fe per 100 cc. of blood. The comparison must be made as quickly as possible, as the acetone evaporates on exposure to the air, although about equally in both cups.

Obtaining Blood.—The ubiquity of iron makes special care to avoid outside contamination necessary in obtaining the blood. The finger or ear lobe should be well cleansed with water and ether before pricking. A free flow, without squeezing, should be obtained. Citrate and oxalate do not interfere with the determination, so that blood drawn by venepuncture and so treated can be analyzed for iron by the method. Corpuscles and plasma should, of course, be well mixed before being drawn into the pipette. If possible, the blood should be drawn in the morning before breakfast in order to have as constant a condition of the blood volume as possible.

The pipette should be calibrated by weighing the water it holds. The blood should always be rinsed into the 2 cc. of water plus 0.2 cc. of 0.1 N HCl. The least amount of blood with which the determination may be made satisfactorily is 0.040 cc.

Hydrobromic Acid.—The hydrobromic acid was the pure concentrated hydrobromic acid, 34 per cent strength. The drops delivered from the pipette were of approximately 0.1 cc. each. If the two drops added to the permanganate coagulum do not clear it up on boiling, another drop may be added to it as well as to the standard.

Permanganate.—The permanganate used should be iron-free, as determined by a blank. If it is not, repeated recrystallization of the commercial product is necessary. Reliable commercial firms, however, furnish an iron-free permanganate. The 0.1 N solution should be kept from the light in a dark bottle.

Standard Iron Solutions.—These are most easily prepared from chemically pure ferric chloride ($FeCl_3.6H_2O$). The standard prepared and used, as proposed by us, contains an amount of iron

equal to that in the hemoglobin of 0.040 cc. of blood when the hemoglobin content is 100 per cent in the Haldane scale; *i.e.*, 13.6 gm. of hemoglobin per 100 cc. of blood binding 18.5 cc. of oxygen. The figure 13.6 gm. of hemoglobin per 100 cc. as a basis may be derived from Hüfner's[10] finding that 1 gm. of hemoglobin will combine with 1.36 cc. of oxygen. This has been confirmed by a number of investigators. If 1 gm. of hemoglobin will combine with 1.36 cc. of oxygen, 18.5 cc. of oxygen will combine with 13.6 gm. of hemoglobin. Butterfield,[11] as others before him, has shown that the iron content of 1 gm. of hemoglobin is constant, lying between 0.33 and 0.34 per cent, that is, one three-hundredth of the molecule. One three-hundredth of 13.6 gives 45 mg. of Fe per 100 cc. of standard blood. This is the standard used in the determination of iron. It may be made up from a concentrated solution of ferric chloride, 4.355 gm. of $FeCl_3.6H_2O$ in a liter of water, by taking 1 cc. and making up to 100 cc. with water, which is the standard solution containing 0.009 mg. of Fe per cc.

If the hemoglobin is much higher than the normal, other standards can be used, corresponding respectively to 25, 35, 55, and 65 mg. of Fe per 100 cc. of blood. In the note on the colorimetric comparison reference will be made to this.

Ammonium Sulfocyanate.—This is prepared as 5 N in order to have a concentrated solution which reduces the degree of dissociation of the ferric sulfocyanate into the colorless ions. It should be kept in a dark bottle away from the light.

Comparison in Colorimeters.—Duboscq and Kober colorimeters proved suitable for this work. A comparison of the standard itself should always be carried out quickly. Readings can be made in daylight, preferably from a north window, or with artificial light filtered through "daylight glass." The average of at least four readings, quickly done before the acetone evaporates, is taken. As in all colorimetric readings, if the reading falls below 12 or above 8 (the standard being at 10) a reading should be made with one of the other standards mentioned above. Indeed, the standard to be used should depend upon the hemoglobin reading; *e.g.*, with 50 per cent hemoglobin the standard corresponding to 25 mg. of Fe per 100 cc. of blood should be used (that is 0.005

[10] Hüfner, G., *Arch. Physiol.*, 1894, 130.

[11] Butterfield, E. E., *Z. physiol. Chem.*, 1909, lxii, 173.

mg. of Fe per cc. of standard iron solution). For every 5 mg. above or below the standard of 45 mg. of Fe per 100 cc., 0.001 mg. is added to or subtracted from the standard of 0.009 mg. of Fe per cc. Ordinarily, the latter standard, corresponding to 45 mg. of Fe per 100 cc., is used.

Accuracy of the Method.—The measurements of the blood and that of the Fe in the standard are the steps upon which the accuracy of the whole method depends (assuming that the colorimetry is satisfactory). They should therefore be executed with extreme care with pipettes calibrated by the analyst. The evaporation of the acetone is guarded against by the employment of long narrow graduates with stoppers. Where evaporation is most disturbing is in the colorimeter cups, where readings must be done quickly and with the liquid at equal levels in both cups.

A number of duplicate determinations on a series of bloods never showed a difference of more than 1 per cent. A series of twenty determinations on the same blood never showed a greater error.

To compare the results of the method with a volumetric method applied to the ash of 5 cc. of blood, that of Wolter[12] was used

TABLE.

Blood sample.	Fe determination.			
	5 cc. blood (volumetric).	0.040 cc. blood (colorimetric).	Difference.	Percentage difference
	mg. per 100 cc.	*mg. per 100 cc.*		
1	48.6	48.2	−0.4	0.82
2	52.4	51.8	−0.6	1.14
3	39.8	40.2	+0.4	1.00
4	46.5	46.4	−0.1	0.23
5	49.6	49.2	−0.4	0.80
6	55.8	55.9	+0.1	0.17

The agreement is close enough to show the accuracy of the method.

SUMMARY.

A method for the determination of iron in as small a quantity of blood as 0.040 cc., using but three simple reagents, in 10 to15 minutes is described.

[12] Wolter, O., *Biochem. Z.*, 1910, xxiv, 108.

I wish to express my obligations to Dr. Herman Schwarz of New York, and to Dr. Donald D. Van Slyke and Mr. J. R. Plazin of the Hospital of The Rockefeller Institute for Medical Research, for their kind suggestions and criticism during the course of the work.

THE DETERMINATION OF BUFFER EFFECTS IN MEASURING RESPIRATION.

By W. J. V. OSTERHOUT.

(*From the Laboratory of Plant Physiology, Harvard University, Cambridge.*)

(Received for publication, June 18, 1918.)

The indicator method of measuring respiration, as described by Haas,[1] has proved useful for a variety of purposes. It consists in placing organisms in a solution containing an indicator and observing changes in color produced by the giving off of CO_2. Since the acidity and color are affected by substances having a buffer action it is evident that when such substances are present the amount of buffer action must be measured. This may be done by means of the apparatus shown in Fig. 1.

The apparatus consists of a capillary tube A, connected by rubber tubing to the Y-tubes B and C (with the thistle tubes, D and E), and provided with the screw clamps F, G, H, and J.

In using the apparatus all the clamps are opened and a stream of CO_2 is allowed to flow in from a generator.[2] After all the air has been driven out of A, B, C, D, and E by the stream of CO_2 the clamp G is closed (while the stream of CO_2 is still running). The clamp F is then closed and mercury is instantly poured into E. The stream of CO_2 now runs through D; while it is still running the clamp H is closed and mercury instantly poured into D.

The clamp F is now cautiously opened, allowing mercury to run into A to any desired distance (raising and lowering E helps to regulate this). The clamp H is then opened, allowing mercury to run in until it becomes visible in A; J is then closed.

[1] Haas, A. R., *Science*, 1916, xliv, 105.

[2] Before entering the apparatus the CO_2 bubbles through saturated aqueous $NaHCO_3$, and then passes through a tower containing glass beads, wet with saturated solution of $NaHCO_3$; this removes HCl. The CO_2 should be allowed to run until it issues from the apparatus free from air. The gas, as it issues from the apparatus, may be tested for the presence of air by collecting it over mercury and introducing NaOH to absorb the CO_2.

The tube C (with its connections) is removed from A, leaving the end of A free and allowing some of the mercury to escape. The CO_2 contained in A is now at atmospheric pressure and is confined by a column of mercury at either end. The length of the column of CO_2 in A is now carefully measured and the temperature noted. If A has been previously calibrated the amount of CO_2 contained in A can be determined.

The free end of A is now thrust into the opening in the stopper S (this opening is too small to permit the escape of the liquid which fills the tube P). In so doing care is taken not to introduce air

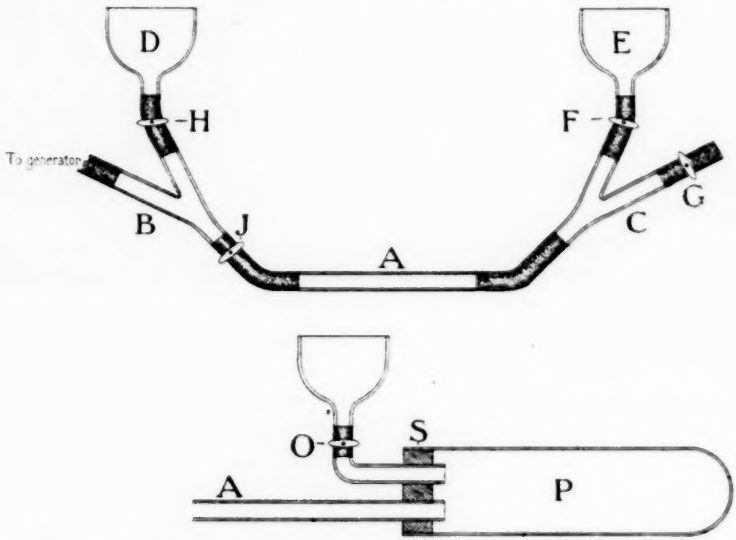

FIG. 1. Apparatus for the determination of buffer effects by adding a measured amount of CO_2 to a known volume of liquid. The CO_2 contained in the tube A is forced into the tube P, which is filled with liquid containing an indicator.

into P (which has previously been filled with liquid in such a manner as to exclude air, the clamp at O being closed soon afterward).

The clamps J and O are now slightly opened and the CO_2 contained in A is forced into P by the pressure of mercury admitted from D (by opening the clamp H) or by squeezing the rubber tubing in the neighborhood of J. By lowering D and by squeezing,

it is possible to admit about the same amount of mercury into P in each experiment. It is desirable to keep the amount as constant as possible.

As soon as this has happened the clamp O is closed. A minute bubble of air may enter with the CO_2 but this may be treated as a constant error and disregarded.

By inclining P, the CO_2 can be made to move back and forth until all of it is dissolved and uniformly mixed with the liquid (the mercury which is admitted with the CO_2 acts as a stirrer).

The liquid in P contains an indicator and its pH value is carefully determined before and after the entrance of the CO_2 by comparing its color with that of a series of buffer solutions[3] (contained in Pyrex tubes of the same size and having the same concentration of indicator). Knowing the volume of liquid in P and the amount of CO_2 introduced, it is easy to determine the change in pH produced by a given amount of CO_2. In order that this change shall not be affected by substances given off by the glass or by the rubber, the rubber should be coated with paraffin which does not give off acid,[1] and the apparatus must be tested by allowing the liquid to stand in it for a period somewhat longer than that of an ordinary experiment to see whether any change in pH occurs. Mercury should then be introduced to see whether it produces any effect; this is not the case if it is pure, but impure mercury may affect the results.

In actual practice it will be found that the relative sizes of A and P may be varied considerably. In order that the stopper S may be sufficiently large the bore of P should be about $\frac{3}{4}$ of an inch.

The apparatus plays an indispensable rôle in the study of respiration by enabling us to ascertain the amount of buffer effect produced by the reagents which are added to the water containing the organisms. For this purpose we first determine the change in pH produced in the water by a given quantity of CO_2; the reagent is then added and a fresh determination is made with approximately the same amount of CO_2. Usually the amount added is not quite the same, owing to difficulties of manipulation; several determinations are therefore made with varying

[3] Cf. Osterhout, W. J. V., and Haas, A. R. C., *Science*, 1918, xliv, 420, footnote 7.

amounts and a curve is drawn from which the desired figures can be obtained by interpolation. When the buffer effect is ascertained it is a simple matter to make a correction for it.

SUMMARY.

In measuring respiration by the indicator method it is necessary to determine the buffer effect of added reagents. This may be done by the apparatus here described, which permits the addition of measured quantities of CO_2 to known volumes of liquid whose pH value is determined before and after the addition of the CO_2.

STUDIES IN CALCIUM AND MAGNESIUM METABOLISM.

V. FURTHER OBSERVATIONS ON THE EFFECT OF ACID AND OTHER DIETARY FACTORS.*

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(Received for publication, June 4, 1918.)

Calcium has long been considered one of the inorganic elements indispensable for the well being of both the adolescent and the adult organism. In the food it is always accompanied by more or less magnesium; but what physiological rôle the latter plays has not been definitely determined. Just recently renewed emphasis has been placed upon the necessity of a suitable supply of calcium in the nutrition of growth.[1] One can now appreciate better than ever the possibility of a deficit of calcium in the pregnant individual and the subsequent importance of lime in the food for the growing young. Consequently adequate information regarding the factors that may influence the metabolism of calcium is highly desirable. Attention has been called to this in the publications of Givens and Mendel[2] and of Stehle.[3]

Givens and Mendel have reported that:

"Administration of base or acid produced no significant effect upon the balance of nitrogen, calcium, magnesium, and phosphorus in the dog.

"Administration of hydrochloric acid increased the urinary excretion of calcium and thereby altered the relation of calcium to magnesium in the urine."

Simultaneously with the appearance of this statement came the conclusion reported by Stehle[3] that:

* A preliminary report appeared in *Proc. Soc. Exp. Biol. and Med.*, 1917–18, xv, 66.

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[1] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1918, xxxiv, 131.

[2] Givens, M. H., and Mendel, L. B., *J. Biol. Chem.*, 1917, xxxi, 421.

[3] Stehle, R. L., *J. Biol. Chem.*, 1917, xxxi, 461.

"The administration of hydrochloric acid by mouth to the dog causes an increased excretion of calcium and magnesium as well as of sodium and potassium but in the case of the latter pair a compensatory retention makes the loss apparent rather than real.

"If an analogous condition holds in human diabetes the resulting calcium loss may be something to take into consideration in the treatment of diabetic patients in whom the excretion of hydroxybutyric acid has reached a significant figure."

Stehle based his conclusions on an experiment conducted upon a dog to which he fed either glycocoll hydrochloride or hydrochloric acid for several days; he then analyzed the excreta. He used a diet decidedly poor in lime—one which was certainly inadequate for the needs of his dog—and drew his conclusions respecting losses on the basis of the extra amount excreted in the acid-feeding periods over that of the fore periods. Stehle did not control his results by a similar experiment on a high lime diet; consequently the reported output of lime may have been augmented by the demand of the tissues for more of this element as the period of shortage increased. In fact the after periods never reached the level of the fore period in respect to the calcium output, but in each succeeding after period it was slightly higher. He rightly states, as his charts show, that ammonia "is the great factor in neutralizing the acid administered."

In an endeavor to obtain an explanation of these apparent discrepancies new experiments have been conducted on two dogs.

Methods.

As in our former study upon this subject the intake and output of calcium, magnesium, and nitrogen were determined and from these data corresponding balances for these elements calculated. The calcium and magnesium determinations in urine were made according to the procedure already outlined.[2] The calcium and magnesium content of the foods and feces was estimated according to McCrudden's directions.[4] Nitrogen was determined in all cases by a Kjeldahl method.

The meats used were bought in large quantities, cleaned free of visible fat, fascia, and tendons, hashed thoroughly by running through an electric meat grinder several times, immediately

[4] McCrudden, F. H., *J. Biol. Chem.*, 1911-12, x, 187.

weighed out in definite quantities, wrapped in paraffined paper, and stored in a frozen state. One lot of cracker meal served for all experiments. The dried skimmed milk was Merrell-Soule Company's "Merso."[5] Analyzed agar-agar was used to afford bulk and insure a daily evacuation of the bowels. Only distilled water was allowed.

The feces were marked off by feeding an adequate dose of carmine intimately mixed with the food of the first meal of each period. This diet was never refused. This mode of administering the marker appears to be preferable to feeding the dye or cork in capsules.

The dogs were catheterized every 72 hours with due antiseptic precautions.

EXPERIMENTAL.

If the excretion of calcium or magnesium is influenced by acid introduced with the food, or contained in the latter in some potential form, or arising in the intestinal tract from alimentary changes, such an outcome ought to be revealed when a fixed acid is administered during a regimen furnishing known intakes of these elements. It does not follow, however, that the effect of acids arising in perverted intermediary metabolism will be entirely comparable with that exerted by acids which gain entrance into the organism through the alimentary path. Thus acids introduced into the gastrointestinal canal may become neutralized and exhibit effects attributable to the neutralization products rather than to the acids *per se*.

In the experiments following, hydrochloric acid has been introduced by sound into the stomach 1 to 1½ hours after meals when the diet was rich and again when it was low in lime. To simulate the possible effect of hydrochloric acid neutralized, the influence of sodium chloride on the excretion was also investigated during a period of high lime feeding.

Table I gives the composition of the diets and the analyses of the various components.

To be sure that the water introduced in giving the dilute acid did not affect the excretion of calcium and magnesium by a

[5] For the dried skimmed milk, "Merso," we are indebted to the Merrell-Soule Co., Syracuse, N. Y.

TABLE I.

Composition of Daily Diets.

Diet.	N	Ca	Mg
	gm.	*mg.*	*mg.*
A (poorer in calcium).....	10.08	78	86
B (richer " ").....	10.58	431	107
B_1 (" " ").....	10.06	474	128

Analyses of the Components of the Diets.

	N	Ca	Mg
	per cent	*per cent*	*per cent*
Meat 1.....	3.57	0.007	0.023
" 2.....	3.36	0.012	0.023
Cracker meal.....	1.66	0.026	0.021
Dried skimmed milk (Merso).....	5.84	1.306	0.122
Agar-agar 1*.....		0.422	0.147
" 2*.....		0.736	0.348

* The discrepancies between these analyses are due to the presence of sand in Agar-agar 1.

resultant increased volume of urine this possibility was tested by giving 200 cc. of water by sound to Dog N on two successive days.

In the acid-feeding periods, 2 gm. of hydrochloric acid per day in a volume of 200 cc. were given by sound. When sodium chloride was introduced it was mixed directly with the food.

The statistics of the experiments are shown in Tables II and III.

Nitrogen.

In the case of Dog M there was a slight rise in the urinary nitrogen when the acid was superimposed upon diet B. With the exception of a slight negative nitrogen balance in this period both dogs were always in a good positive nitrogen balance. Consequently we may conclude that neither acid nor sodium chloride, in the amounts here introduced, has a significant effect upon the nitrogen excretion of the dog.

Calcium.

On the acid-free diets A, B, and B_1, whether low or high in lime, Dog M had an average daily urinary excretion of 10 to 12 mg. of

calcium. Dog N did not show such a constant output on the same diets, the calcium in the urine varying from 15 to 34 mg. per day. This discrepancy between two animals of the same species is comparable to the variations noted between different men.[6]

When Dog M on the low lime diet A received 2 gm. of hydrochloric acid per day for a period of 12 days its daily excretion of calcium increased to 23 to 28 mg.; that is, over 100 per cent. The response in increased urinary lime was prompt and constant. When the acid in the same amount and over the same period of time was introduced during the high lime dietary period there was an increased urinary excretion; but the rise was gradual—14 mg. on the 1st day and 30 mg. on the 12th day or increases of 16 and 150 per cent respectively above the average normal.

TABLE II.

Nitrogen, Calcium, and Magnesium Metabolism.
Dog M. Averages per Day.

Period.	Days.	Weight.	Intake.		Urine.			Feces.			Balance.		
			Standard diet.	Daily additions.	N	Ca	Mg	N	Ca	Mg	N	Ca	Mg
		kg.			*gm.*	*mg.*	*mg.*	*gm.*	*mg.*	*mg.*	*gm.*	*mg.*	*mg.*
I	3	21.5	A		8.21	9	38						
	3		"		8.26	10	40	0.94	325	65	+0.9	−256	−18
II	3	21.3	"	2 gm. HCl	8.94	23	45						
	3		"	2 " "	8.83	25	40						
	3		"	2 " "	9.03	28	32						
	3		"	2 " "	9.14	28	40	0.71	256	49	+0.4	−204	−2
III	3	21.2	B		9.28	13	33						
	3		"		9.46	11	34	0.71	570	81	+0.5	−151	−8
IV	3	20.9	"	2 gm. HCl	10.3	14	39						
	3		"	2 " "	10.4	21	35						
	3		"	2 " "	10.1	21	33						
	3		"	2 " "	9.83	30	34	0.77	654	88	−0.3	−244	−16
V	3	20.0	"		9.74	11	19						
	3		B_1		8.86	12	40						
	3		"		8.78	11	39	0.80	643	84	+0.3	−199	+5
VI	3	20.5	"	3.21 gm. NaCl	8.64	16	47						
	3		"	6.42 " "	8.83	17	41						
	3	20.2	"	9.63 " "	8.82	21	44	0.79	608	82	+0.5	−152	+2

[6] Givens, M. H., *J. Biol. Chem.*, 1918, xxxiv, 119.

TABLE III.

Nitrogen, Calcium, and Magnesium Metabolism.
Dog N. Averages per Day.

Period.	Days.	Weight.	Intake.		Urine.			Feces.			Balance.		
			Standard diet.	Daily additions.	N	Ca	Mg	N	Ca	Mg	N	Ca	Mg
		kg.			*gm.*	*mg.*	*mg.*	*gm.*	*mg.*	*mg.*	*gm.*	*mg.*	*mg.*
I	3	23.5	A		9.58	34	32						
	3		"		8.50	26	26	0.54	355	69	+0.5	−307	−13
II	3	23.5	"	200 cc. H_2O	8.06	27	27	0.60	351	71	+1.4	−299	−12
III	3	23.5	"	2 gm. HCl	8.32	55	36						
	3		"	2 " "	9.15	58	33						
	3		"	2 " "	9.07	58	34	0.45	312	58	+0.8	−291	−6
IV	3	23.2	B		8.75	26	26						
	3		"		9.59	19	26	0.53	671	94	+0.9	−263	−13
V	3	23.0	"	2 gm. HCl	10.2	24	31						
	3		"	2 " "	10.0	47	34						
	3		"	2 " "	9.76	49	31						
	3		"	2 " "	10.5	47	35	0.45	656	91	0.0	−267	−17
VI	3	22.3	"		9.76	23	29						
	3		B_1		9.60	19	36						
	3		"		9.03	15	34	0.51	645	93	+0.3	−205	−6
VII	3	22.2	"	3.21 gm. NaCl	8.76	18	32						
	3		"	6.42 " "	9.04	31	30						
	3	22.1	"	9.63 " "	8.88	41	25	0.47	627	95	+0.7	−183	+4

When sodium chloride equivalent to the amount of acid given in the experiments was mixed with the food of diet B_1, the daily calcium excretion increased from 11 mg. to 16 mg. on this salt régime. When double the amount of salt was added to the diet, there was a further slight rise, and likewise a still further increased output of calcium when three times as much salt was fed. In the last period of highest salt intake there was a slight diuresis but not sufficient to account for the increased calcium output. Judging by our analyses of human urines we doubt whether any ordinary diuresis will appreciably increase the output of the alkali earths.

It will be observed from the tables that comparable results were obtained upon Dog N. In as far as the increased urinary output of calcium, after acid introduction, is concerned these results

confirm those previously reported by Givens and Mendel and by Stehle.

Inasmuch as the fecal excretion represents both absorbed and reexcreted calcium it is practically impossible to disentangle these factors by an analysis of the feces collected in a given period. The individual periods of collection have been made long—usually 6 to 12 days—to make the balances more representative.

On the diet low in lime the acid provoked a decreased fecal excretion of calcium in the case of Dog M; but had practically no effect on Dog N. Accordingly the calcium balance was favorably influenced by the acid in one case and not affected by it in the other.

When the intake of lime was higher the figures for the fecal excretion of calcium during acid ingestion for Dog M are larger than in the fore period but of the same magnitude as those of the after period. This cannot be ascribed to a so called lag as the after period was 9 days in duration. It seems more plausible to assume that in the fore period on the high lime intake the animal was trying to store up some lime after its depletion by the former low lime régime. The sodium chloride has, if anything, slightly decreased the fecal loss of lime. Dog N, on the other hand, showed no marked change in the fecal excretion of lime under any of the dietary alterations, although there are indications that the acid and salt have tended to decrease the lime excretion through the feces in this animal.

The balances show that, on the high lime diet, the acid had a slightly unfavorable influence upon one animal, but hardly affected the other. These results confirm our former statement *that administration of acid produces no significant effect upon the balance of calcium in the dog.* They do not lend support to Stehle's conclusion.

Magnesium.

The variations in the urinary magnesium excretion of both dogs on either of the diets were so slight that it appeared that neither the acid nor the salt introduced into the organism had any effect upon the elimination of this element by the kidneys. The fecal excretion of magnesium likewise was not appreciably affected by any procedure.

The average daily magnesium balance of both animals in most of the periods was slightly negative. Where it was on the plus side the gain was so small as to be without advantage.

Calcium-Magnesium Relationship.

Dog M always excreted by way of the kidneys more magnesium than calcium. The relationship between the elements was only altered by the increased urinary calcium in the acid periods. The fecal excretion of calcium, of course, always *decidedly* preponderated over that of the magnesium.

In the case of Dog N the relation of calcium to magnesium approached 1:1 unless the urinary calcium output was increased by either acid or sodium chloride. The ratio of calcium to magnesium in the feces was always decidedly in favor of the calcium, as in the other dog.

DISCUSSION.

It does not seem permissible to make decisive statements regarding the influence of any substance upon the excretion of the alkali earths unless the outcome has been very *marked*. If it were practicable to carry out feeding experiments with these elements without the possibility of a reexcretion after absorption then one could make statements with more certainty.

On a low lime intake the administration of acid promptly increased the excretion of calcium *by the kidneys*. Such was not the case when the diet contained an amount of lime which approached the requirement of the animal; there was a response at once, but it took several days for the amount of the excreted element to reach the level of the first acid period on the low lime intake. A temporary protection was evident.

With both dogs on the low lime diet the output of calcium, *by way of the intestines*, was apparently slightly decreased by the ingestion of acid. On the high lime intake during acid administration, Dog M showed an increased output of calcium if we judge by the fore period, whereas from the after period the indications are that the fecal excretion of lime was not influenced by the acid. This idea is strengthened by the fact that the output in the period following the after period was of about the same magnitude.

It seems likely that in the fore period the dog was trying to store up some lime after having been on the low lime intake for so long a time.

With Dog N the fecal excretion of calcium on the high lime diet during acid ingestion was scarcely altered.

If any conclusions are to be drawn regarding the influence of acid ingestion upon the excretion of alkali earths it seems most logical to depend on balance experiments and not upon a comparison of the fecal outputs or data in which the fore period or after period is taken as a basis, as Stehle has done.

The negative calcium balance in the case of Dog M on the diet low in lime was reduced by acid administration. In this dog, when the intake of calcium was increased, the acid appears to have increased the negative calcium balance; while the administration of sodium chloride slightly reduced the negative balance of lime.

In Dog N the acid did not alter the calcium balance during the low lime feeding. On the increased lime intake the superimposed acid has not affected the balance of calcium if the fore period is used as a basis for comparison, whereas if the after period is used, the acid did slightly increase the negative calcium balance. The later comparison does not seem justifiable, for if the acid is capable of exerting an influence it certainly ought to have done so in a period of 12 days. The sodium chloride apparently reduced the negative calcium balance, as it did in the case of Dog M.

If calcium plays any rôle at all as a neutralizing agent in the acidosis provoked by introduction of a mineral acid it is at best a very small one. The store of calcium in the organism is in the bones and not in the soft tissues. To affect this store a very large dose of acid must be used. Thus Steenbock, Nelson, and Hart[7] did not consider that they had affected the bones of a calf until they gave it as much as 400 cc. of normal hydrochloric acid daily. They thought then that only the calcium carbonate of the bones was affected and not the calcium phosphate, as the phosphorus in the urine was not increased. When doses of acid comparable to those employed by Stehle or ourselves were used

[7] Steenbock, H., Nelson, V. E., and Hart E. B., *J. Biol. Chem.*, 1914, xix, 399.

they found the neutralization to be accomplished by ammonia. Many other experiments[8] could be cited showing that ammonia is the dominant factor in neutralizing acid introduced or formed during metabolism, but the point has been demonstrated in so many experiments on man and animals that it is now a generally accepted fact.

Albu and Neuberg[9] state that the degree of calcium absorption depends in part upon what other salts are taken with the nourishment; especially is it claimed that sodium chloride increases the absorption of calcium. Concerning the effect of sodium chloride on calcium excretion we have been able to find in the literature only one direct experiment. Von Wendt[10] after feeding this salt to man in increasing amounts on a known diet received on the corresponding days a progressive increase in the urinary excretion of calcium. In this respect our experiments on dogs correspond with his findings. The unfavorable influence on the *balance* of calcium after sodium chloride feeding, reported by von Wendt, has not been duplicated in our observations.

SUMMARY.

As a result of new experiments we are able to confirm our previous statement that ingested hydrochloric acid is without marked influence on the calcium and magnesium metabolism of the dog. The previously reported increased urinary elimination of calcium after acid consumption has been observed anew; but the absolute amount of this increase was not sufficient to affect to any noticeable degree the calcium balance.

[8] Concerning the rôle played by ammonia in neutralizing acid, see: in the case of the dog, Eppinger, H., *Z. exp. Path. u. Therap.*, 1906, iii, 530; Eppinger, H., and Tedesko, F., *Biochem. Z.*, 1909, xvi, 207; Secchi, R., *ibid.*, 1914, lxvii, 143; in the rabbit, Walter, F., *Arch. exp. Path. u. Pharmakol.*, 1877, vii, 148; Winterberg, H., *Z. physiol. Chem.*, 1898, xxv, 202; Eppinger, *Wien. klin. Woch.*, 1906, xix, 111; Secchi, *loc. cit.*; in the pig, McCollum, E. V., and Hoagland, D. R., *J. Biol. Chem.*, 1913-14, xvi, 299; in the sheep, Eppinger and Tedesko, *loc. cit.*; in man, Secchi, *loc. cit.*, Begun, A., Hermann, R., and Münzer, E., *Biochem. Z.*, 1915, lxxi, 255.

[9] Albu, A., and Neuberg, C., Physiologie und Pathologie des Mineralstoffwechsels, Berlin, 1906.

[10] von Wendt, G., *Skand. Arch. Physiol.*, 1905, xvii, 211.

The magnesium balance and the partition of this element between the different paths of excretion was not significantly altered.

Sodium chloride (which might arise through the neutralization of ingested hydrochloric acid) was fed in increasing amounts and produced a corresponding increase of calcium in the urine without noticeably affecting the balance of calcium. It was without any decided effect upon the excretion of magnesium.

My thanks are due Professor Lafayette B. Mendel for his criticisms.

THE PROPERTIES AND COMPOSITION OF OOCYTIN.*

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In 1908 Loeb (1) discovered that mammalian blood serum possessed the property of inducing the formation of fertilization membranes and the initial stages of development in sea urchin eggs which had previously been immersed in $SrCl_2$ solution. He also showed that the active substance is precipitable by acetone and destroyed by heating.

In 1912 Robertson (2) reported the isolation of a small fraction from mammalian blood serum which he designated "oocytin" and which he found to possess the same power of inducing the formation of fertilization membranes as the whole serum.

To obtain sufficient material for a more complete study it was necessary to work over large quantities of blood. Through the courtesy of the Oakland Meat and Packing Company it was possible to obtain an unlimited supply of fresh, defibrinated beef blood. The yield of serum from defibrinated blood varies greatly with different animals, centrifuging for an hour, at a rate of 3,000 revolutions per minute, yielding from 25 to 40 per cent of serum.

From a series of preliminary experiments it was found that the following procedures gave the maximum yield of oocytin:

1. The defibrinated blood and serum are kept cool (in a refrigerator).

2. 100 cc. of 10 per cent barium chloride solution are added, with constant stirring, to each liter of serum. This mixture is also kept in a cool place for 48 hours, when the supernatant fluid can be decanted. The residue is washed several times with large volumes of 2 per cent barium chloride solution to remove all traces of serum.

3. The residue from above is stirred with 0.1 N HCl (warmed to 45°C.), using 50 cc. of acid for each liter of serum. After stirring for an hour or

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more the mixture is centrifuged and to the clear liquid thus obtained, an equal volume of 0.1 N H_2SO_4 is added. This solution is allowed to stand in a warm place (40°C.) for 24 hours and then thoroughly centrifuged to remove $BaSO_4$. (The solution should contain an excess of sulfate ions.)

4. Four to five volumes of best grade acetone are now added to the acid solution and the mixture is put in a cool place for 8 hours or more. The acetone-acid solution is then siphoned off, the precipitate collected on a hardened filter, washed with absolute alcohol, and finally with anhydrous ether. The ether-moist precipitate is placed in an incubator containing H_2SO_4 and dried for 48 hours at 38°C.

TABLE I.

Elementary Composition of Oocytin.

Sample.	Date of preparation.	Carbon.*	Hydrogen.*	Nitrogen.†	Oxygen by difference	Ash.	Phosphorus	Sulfur.‡
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
B	July, 1917	32.26	6.44	9.46	33.50	21.2 { 1.94 Ba, 7.27 Ca }	0.47	—
A	Aug. "	39.05	7.57	12.81	31.58	8.8 { 2.68 Ba, — Ca }	—	—
H	Feb., 1918	31.62	9.09	7.63	26.09	25.6 { 1.73 Ba, 7.56 Ca }	—	—
I	" "	29.77	8.15	8.79	33.68	21.0 { 2.34 Ba, 6.79 Ca }	—	—
J	Mar. "	—	—	9.46	—	16.0 { 0.56 Ba, 9.37 Ca }	—	6.10

* The determinations of carbon and hydrogen were carried out according to the method recently described by Levene and Bieber (3).

† The nitrogen was determined by direct Nesslerization, using Folin's (4) micro method.

‡ The sulfur was determined by sodium peroxide fusion, using an alcohol lamp.

The above procedures differ from those employed by Robertson (2) in the following particulars: (1) Only one-fifth as much barium chloride solution was used. (2) No NaCl solution was added to the serum to make an isotonic solution before the addition of the $BaCl_2$ solution. (3) Using H_2SO_4 instead of Na_2SO_4 to precipitate the excess of barium.

The material obtained by the above procedures is a white, slightly hygroscopic powder, soluble in dilute acids. The yield varies considerably. From the 130 gallons of blood used, an average of 0.7 to 0.85 gm. per 10 gallons of blood was obtained. Several samples were prepared during the year, all of which gave the same qualitative tests and color reactions but which varied widely in elementary composition. Table I contains the results of the quantitative analyses of five samples. These results show clearly that oocytin, thus prepared, is not a chemical individual.

The most available method of ascertaining the relative purity of the several samples and of identifying them with the material prepared by Robertson (2) was to determine their action upon sea urchin eggs (*Strongylocentrotus purpuratus*). The results of this work are presented in Table II. It is very evident that heating to 80°C. (Sample E) for 5 minutes completely destroys the active constituent of oocytin. Loeb (5) found that the active substance present in blood serum was not affected by heating to 73°C. for 30 minutes, but that boiling for a few minutes completely destroyed it. Robertson (2) showed that a temperature of 58°C., maintained for 18 hours, had no injurious effect upon the active substance in blood serum. It is therefore evident that the substance capable of inducing membrane formation and cytolysis in sea urchin eggs is destroyed at a temperature lying between 73 and 80°C. Contact with alcohol (Sample D) for a number of days also destroys the active constituent. From a comparison of the action of Samples A, B, F, and G it is evident that the samples containing large amounts of barium are not as effective as the purer samples in inducing membrane formation. That contamination with barium is not the cause of membrane formation and cytolysis is also apparent from the negative results obtained with Sample D, exposed to alcohol, and Sample E, heated to 80°C. Since the action of Sample G, prepared by Robertson (2) in 1912, compares very favorably with that of the purest obtained (Sample A), it is evident that the active substance is not affected by prolonged exposure to air and light.

TABLE II.—*Effects of Oocytin Upon Sea*

Dilution of oocytin solution.†	Sample A,† purest preparation obtained (2.68 per cent Ba).	Sample B,‡ contains 18 per cent Ba.	Sample C,‡ in contact with acetone for 30 days.
1 : 800	In 12 min. 10 per cent of closely formed membranes. No clumping. In 45 min. clumping and fusion of membranes in the clumps. In 78 min. 80 per cent of regular membranes. In 124 min. no further change.	In 9 min. clumping and cytolysis. In 120 min. eggs surrounded by dense precipitate. In 295 min. most of clumps resolved, about 30 per cent of normal membranes.	In 10 min. clumping very marked. In 312 min. most of eggs in closely packed clumps and surrounded by dense precipitate.
1 : 12,800	In 18 min. small amount of cytolysis, no clumping. In 287 min. about 5 per cent of imperfect, blistery membranes, most of eggs cytolyzed.	In 30 min. eggs normal, some clumping. In 57 min. few imperfect membranes.	In 52 min. few imperfect membranes. Considerable cytolysis.
1 : 102,400	In 40 min. few membranes, some cytolysis, no clumping. In 105 min. 3 per cent of imperfect membranes, many eggs cytolyzed.	In 50 min. few imperfect membranes, some cytolysis, and clumping.	In 68 min. about 20 per cent of imperfect membranes, some cytolysis.
1 : 204,800	In 73 min. an occasional membrane, few cytolyzed eggs, others normal.	In 114 min. about 15 per cent of imperfect membranes and 10 per cent of eggs cytolyzed. In 302 min. the cytolysis is much more pronounced.	In 319 min. about 5 per cent of well formed membranes, some cytolysis.
1 : 409,600	In 55 min. few perfect membranes, some cytolysis. In 99 min. no further change. In 271 min. about 20 per cent of regular membranes.	In 109 min. few cytolyzed eggs, others normal. In 308 min. about 5 per cent of imperfect membranes.	No observations made.

* All eggs sensitized by immersion in 0.37 M strontium chloride solution.

† The oocytin solution was prepared by dissolving the desired amount in 0.1 N acid, adding an equal volume of 0.1 N alkali, and made up to necessary volume with NaCl solution of such concentration that the final volume was isotonic with sea water. Other dilutions of oocytin were prepared by diluting with filtered sea water.

*Urchin (Strongylocentrotus purpuratus) Eggs.**

Sample D,‡ in contact with alcohol 90 days.	Sample E,‡ accidentally heated to 80° C. for 5 minutes.	Sample F,§ same as Sample A.	Sample G,§ prepared by Robertson (2) in 1912.
In 15 min. eggs normal except for a slight cytolysis.	In 5 min. clumping very pronounced. In 88 min. most of clumps resolved. Eggs normal.	In 8 min. few perfect membranes, some clumping. In 61 min. no further change.	In 6 min. clumping very pronounced. In 29 min. about 30 per cent of membranes, difficult to determine because of clumping.
No observations made.	In 25 min. eggs normal.	In 50 min. about 10 per cent of regular membranes. Considerable cytolysis and clumping.	In 55 min. 20 per cent of membranes, clumping very pronounced.
In 60 min. few cytolyzed eggs, others normal.	In 84 min. eggs normal.	In 14 min. 40 per cent of regularly formed membranes, some clumping.	In 18 min. few membranes, marked clumping, some cytolysis.
In 130 min. cytolysis of many eggs.	In 82 min. eggs normal.	In 24 min. 15 per cent of well formed membranes, some cytolysis, clumping. In 116 min. no further change.	In 76 min. about 25 per cent of membranes, much clumping.
No observations made as no evidence that material is active.	In 65 min. eggs normal.	In 38 min. 20 per cent of membranes, some cytolysis and clumping. In 67 min. 45 per cent of membranes. In 120 min. no further change.	In 34 min. about 5 per cent of membranes, other eggs normal.

‡ Experiments with Samples A to E, inclusive, were made with the mixed eggs of six female urchins, temperature during the day being 14–16°C.

§ Experiments with Samples F and G were made on the following day with the eggs of one female urchin. Temperature 20°C. Controls were made each day with sensitized and unsensitized eggs in sea water alone.

Qualitative Tests and Color Reactions.

The following tests were made with nine different preparations of oocytin:

Millon's Test.—All give an atypical red precipitate, the color fading on continued boiling. This test indicates the presence of the tyrosine group. This group was also indicated by Folin's (6) phosphotungstic-phosphomolybdic reagent.

Xanthoproteic Test.—All strongly positive.

Biuret Test.—All positive, the color being bluish violet.

Adamkiewicz' Test.—All positive. This test and the two following indicate the presence of the tryptophane group.

Acree-Rosenheim Test.—All positive.

Hopkins-Cole Glyoxylic Acid Test.—All positive.

Molisch's Test.—All positive.

Five different samples were hydrolyzed with 5 per cent H_2SO_4 for 24 to 36 hours, the liquid was filtered, and the following tests were applied:

Molisch's Test.—All very strongly positive.

Bial's Pentose Test.—All give characteristic green color.

Fehling's Test.—All negative.

Nylander's Test.—All negative.

Selivanoff's Test.—All negative.

Benedict's Qualitative Glucose Test.—A very slight reduction, the precipitate being a light brown color.

Ammoniacal Silver Nitrate Test.—A brown, flocculent precipitate of purine bases is obtained. Attempts to obtain the typical silver nitrate salts resulted only in a few characteristic crystals of hypoxanthine silver nitrate.[1]

Phenylhydrazine Test.—No reaction.

Wheeler-Johnson Pyrimidine Test.—Negative.

Portions of the solutions of two of the hydrolyzed samples were evaporated to one-half of their original volume and from the syrupy liquids a few long, colorless crystals were obtained. Examination under the microscope showed them to be identical in form with those of *l*-α-proline.[2]

Attempts were made to prepare the copper salt and the picrate of proline but with the small amount of material available these attempts were not successful.

[1] Hawk, P. B., Practical physiological chemistry, Philadelphia, 5th edition, 1916, 351.

[2] Hawk,[1] p. 84.

Experiments[3] were carried out to determine whether oocytin possessed the property of hemolyzing red cells.

For this purpose 0.1 to 0.5 cc. of oocytin solution[4] (dilution of 1:8,000, this being twice the concentration in which it is obtained from blood serum) was added to tubes containing 0.5 cc. of 3 per cent suspension of washed sheep cells; the tubes were then incubated for 30 minutes at 38°C. No hemolysis was observed. An experiment was also carried out to determine whether oocytin could take the place of alexin in the alexin fixation reaction. For this purpose 0.1 to 0.5 cc. of oocytin solution[4] was added to a hemolytic system in which the alexin was omitted. No hemolysis was observed. A control test in which the alexin was added showed hemolysis in the usual time. The above results indicate that, for the concentration used, oocytin cannot replace alexin in the fixation reaction. The fact that oocytin resists heating to 73°C. is also rather conclusive evidence against its having any identity with alexin. To determine whether oocytin possessed bactericidal properties, 0.1 to 0.5 cc. of each of several concentrations of oocytin solution[4] (1:16,000, 1:1,600, 1:320, 1:160) was added to 0.1 cc. of 24 hour typhoid broth culture (dilution of 1:500,000) and incubated for 18 hours at 37°C. Using the same amounts of the various concentrations of oocytin, experiments were also carried out to determine whether oocytin, in conjunction with inactivated serum (serum heated for 30 minutes at 56°C.), possessed bactericidal properties.

The results of these experiments indicate that oocytin does not possess bactericidal properties and cannot reactivate heated serum. A control experiment, using 0.1 cc. of normal serum with the same typhoid culture, showed bactericidal action.

Alexin fixation experiments[5] were carried out with the sera of Rabbits 161 and 166. No. 166, weighing 1.4 kilos, received six intravenous injections of 50 mg. each. No. 161, weighing 2 kilos, received six intravenous injections of 75 mg. each. The solutions of oocytin were made by dissolving in 0.1 N HCl, adding an equal amount of 0.1 N NaOH, and made up to a volume of 5 cc. by the addition of isotonic NaCl solution. The doses were administered on 3 successive days, then a resting period of 5 days, followed by 3 more successive doses. On the 10th day after the last dose the animals were bled and the serum was used for the fixation experiments. A control

[3] The experimental work to determine whether oocytin could replace alexin or whether it possessed hemolytic or bactericidal properties was carried out by Miss Ruth L. Stone, of the Department of Pathology.

[4] All solutions of oocytin were prepared by dissolving sample in 0.1 N acid, adding an equivalent volume of 0.1 N alkali, and made to desired volume by adding sufficient NaCl solution to make isotonic (M/16).

[5] The fixation experiments were carried out by Dr. Carl L. A. Schmidt, of the Department of Pathology.

experiment, using the serum of a normal rabbit, was conducted at the same time. Using 0.1 cc. of a 1:500 dilution of the antigen, which was one-fourth of the minimum inhibiting dose (solution of antigen, oocytin, was made in the same way as the solutions for injection), and 1.5 units of alexin, with a total volume of 1 cc. for the system, fixation was obtained with 0.2 cc. of a 1:10 dilution for Rabbit 161 and 0.3 cc. of similar dilution for Rabbit 166. The control was negative.

Intravenous injection of 40 mg. of oocytin per kilo produces no change in the blood pressure of rabbits

TABLE III.

Amino Nitrogen Content of Oocytin before and after Hydrolytic Decomposition (Van Slyke Method).*

Sample.	Date of preparation.	Amount of amino nitrogen before hydrolysis, as total N.	Amino nitrogen after complete hydrolysis, as total N.
		per cent	*per cent*
B	July, 1917	20.51	57.30
A	Aug., "	11.15	62.95
J	Mar., 1918	14.93	65.85

* The variation in the amount of amino nitrogen in oocytin, previous to hydrolytic decomposition, is no doubt due to the action of the 0.1 N acid in which the material is originally dissolved.

From the several color reactions, the amino nitrogen content, and the formation of antibodies it is evident that protein material is a constituent of oocytin. Since it has been shown by Robertson (2) that proteins, far from facilitating membrane formation, actually inhibit the action of spermatozoa and saponins in causing membrane formation and cytolysis in sea urchin eggs, it does not seem plausible to ascribe the action of oocytin to its protein content.

The presence of a carbohydrate group is certain; that it is a pentose also seems quite definite. The test with Bial's orcinol-$FeCl_3$ reagent is substantiated by Tollen's (7) naphthoresorcinol test. Mandel and Neuberg (8) have pointed out that other substances may give the same reaction with this reagent as the pentoses. The color reaction with glucuronic acid (indicating hexose) is very similar but this substance should show characteristic absorption bands near the D line of the spectrum. No absorption bands were visible in the two solutions (ether extract of the reaction mixture) examined.

The presence of a carbohydrate group and purine bases in the decomposition products would suggest the presence of nucleic acid but this is contraindicated by the very low phosphorus content. Levene and Jacobs (9) obtained a substance, by ferment action, from animal nucleic acid which on hydrolysis yielded guanine and a hexose. This combination is known as a nucleoside. These workers have also obtained other nucleosides (guanosine, adenosine, cytidine, uridine) by neutral hydrolysis of yeast nucleic acid and find that these nucleosides are composed of a carbohydrate group and a purine or pyrimidine base. From their behavior it is thought that the purine nucleosides are joined through a glucosidal linkage. Levene and Jacobs (10) found guanosine in the pancreas and Jones (11) found that extract of pancreas liberates phosphoric acid quantitatively from guanylic acid, resulting in guanosine and phosphoric acid. According to Levene and Medigreceanu (12) there are at least three ferments involved in the decomposition of the nucleic acids by the body tissues and tissue fluids. Jones (11) has shown the independent existence of a phosphonuclease, yielding nucleosides and phosphoric acid, and a purine nuclease which splits off purine bases.

The presence of nucleosides (purine bases and pentose) and their glucosidal nature suggest that the action of oocytin may be due to these bodies. In this connection reference may be made to the saponins which are complex glucosides. These substances have been shown by Loeb (13) to be very potent in causing membrane formation and cytolysis in sea urchin eggs. The acid-soluble substance prepared from the NaCl solution extract of sea urchin sperm, precipitable by $BaCl_2$ and acetone, is also very potent in its membrane-forming and cytolyzing action. Some of this substance, which had been prepared by Robertson (2) in 1912, was examined and among the hydrolytic products purine bases and a carbohydrate group (pentose) were found, again suggesting the presence of nucleosides.

SUMMARY.

1. The elementary analysis shows that oocytin, prepared as described, is not a chemical individual.

2. Protein material is a constituent of oocytin.

3. The products of hydrolysis of oocytin include purine bases (hypoxanthine) and a carbohydrate radical (pentose). Phosphoric acid is, however, only present in insignificant traces.

4. Oocytin is neither hemolytic, bactericidal, nor identical with alexin.

5. The active constituent is destroyed by heat at a temperature between 73–80°C., and by prolonged contact with alcohol.

6. The fertilizing and cytolytic action of oocytin is attributed to the glucosidal structure of the nucleosides, which are probably the active constituents of the preparation.

In conclusion the writer wishes to thank Professor T. Brailsford Robertson, Professor Hardolph Wasteneys, and Dr. Carl L. A. Schmidt for their helpful suggestions and interest in the work.

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3. Levene, P. A., and Bieber, F. W., *J. Am. Chem. Soc.*, 1918, xl, 460.
4. Folin, O., and Denis, W., *J. Biol. Chem.*, 1916, xxvi, 473.
5. Loeb, *Arch. ges. Physiol.*, 1908, cxxiv, 37.
6. Folin and Denis, *J. Biol. Chem.*, 1912, xii, 239, 245.
7. Tollens, B., *Ber. chem. Ges.*, 1908, xli, 1788.
8. Mandel, J. A., and Neuberg, C., *Biochem. Z.*, 1908, xiii, 148.
9. Levene, P. A., and Jacobs, W. A., *J. Biol. Chem.*, 1912, xii, 377.
10. Levene and Jacobs, *Biochem. Z.*, 1910, xxviii, 127.
11. Jones, W., *J. Biol. Chem.*, 1911, ix, 129, 169.
12. Levene, P. A., and Medigreceanu, F., *J. Biol. Chem.*, 1911, ix, 65, 375.
13. Loeb, Die chemische Entwicklungserregung des tierischen Eies, Berlin, 1909, 135.

CONCERNING HISTIDINE AND CARNOSINE. THE SYNTHESIS OF CARNOSINE.*

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HISTORICAL.

Our knowledge of carnosine is largely due to the admirable investigations of Gulewitsch. The discovery of the base in beef muscle extract was first reported by Gulewitsch and Amiradžibi[1] in 1900. Later Kutscher[2] isolated a substance from beef muscle extract, which he named ignotine, but Gulewitsch[3] showed that ignotine and carnosine were identical. In the same volume, he[4] showed that hydrolysis of the base with barium hydroxide liberated the amino-acid, histidine. 5 years later, he[5] established the identity of the other component, namely β-alanine. This amino-acid had been found in beef extract by Engeland,[6] as a result of the hydrolysis of carnosine during the process of isolation. Thus 11 years after its discovery, Gulewitsch showed that carnosine consisted of but two amino-acids, probably joined to form a dipeptide. Either one of two linkages was possible. Thus far no conclusive evidence had been advanced in favor of either formula.

On the whole little has been done to establish the relative distribution, biological significance, and fate of carnosine in the

* The data are taken from a dissertation submitted by Thorsten Ingvaldsen to the Graduate Faculty of the State University of Iowa, as a partial requirement for the degree of Doctor of Philosophy.

[1] Gulewitsch, W., and Amiradžibi, S., *Z. physiol. Chem.*, 1900, xxx, 565; *Ber. chem. Ges.*, 1900, xxxiii, 1902.

[2] Kutscher, F., *Z. Untersuch. Nahrungs- u. Genussmittel*, 1905, x, 528.

[3] Gulewitsch, W., *Z. physiol. Chem.*, 1906–07, l, 204.

[4] Gulewitsch, *Z. physiol. Chem.*, 1906–07, l, 535.

[5] Gulewitsch, *Z. physiol. Chem.*, 1911, lxxiii, 434.

[6] Engeland, R., *Z. Untersuch. Nahrungs- u. Genussmittel*, 1908, xvi, 658.

body. Gulewitsch prepared the base by precipitation of muscle extract with phosphotungstic acid, followed by purification by the silver-barium fractionation method of Kossel and Kutscher. The nitrate of carnosine was thus obtained in crystalline form. Later Smorodinzew[7] precipitated carnosine with mercuric sulfate in sulfuric acid solution. This author also demonstrated the absence of carnosine in the liver. Dietrich[8] recommended the addition of large quantities of alcohol to accelerate the precipitation with mercuric sulfate.

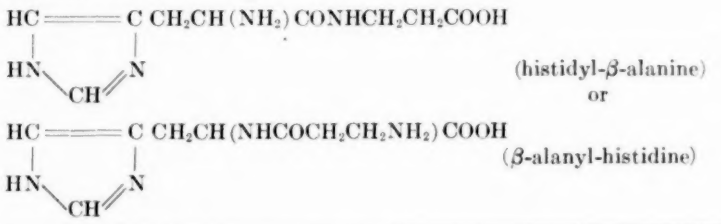
Skworzow,[9] it appears, first attempted the quantitative determination of carnosine by estimating the nitrogen content of the carnosine silver fraction. He found that 35 and 51 per cent of the nitrogen precipitated by phosphotungstic acid from calf and beef muscle extracts, respectively, appeared in the carnosine silver fraction, but did not attribute all of this to carnosine itself. He actually isolated an amount of carnosine nitrate corresponding to 0.176 per cent of the free base from calf muscle extract. This value agrees with those obtained by other investigators. Von Fürth and Schwarz[10] found that carnosine accounted for from 30 to 44 per cent of the total extractive nitrogen of the skeletal muscle of the horse and dog. Buglia and Costantino[11] and von Winiwarter[12] applying similar methods concluded that about one-third of the extractive nitrogen contained in skeletal muscle was in the form of carnosine. Miss Mauthner[13] unsuccessfully attempted the quantitative isolation of carnosine as the copper salt, which had been prepared previously by Gulewitsch. She next hydrolyzed the carnosine silver fraction and was able to isolate from 70 to 90 per cent of the theoretical quantity of histidine as the picrolonate (assuming that carnosine wholly accounted for the nitrogen content of this fraction). Miss Mauthner also succeeded in precipitating 80 per cent of the nitrogen contained in the carnosine silver fraction, as the sodium salt of carnosine dipicrolonate.

[7] Smorodinzew, J., *Z. physiol. Chem.*, 1914, xcii, 214.
[8] Dietrich, M., *Z. physiol. Chem.*, 1914, xcii, 212.
[9] Skworzow, W., *Z. physiol. Chem.*, 1910, lxviii, 26.
[10] von Fürth, O., and Schwarz, C., *Biochem. Z.*, 1911, xxx, 413.
[11] Buglia, G., and Costantino, A., *Z. physiol. Chem.*, 1912, lxxxi, 120.
[12] von Winiwarter, A., *Arch. Gynäk.*, 1913, c, 530.
[13] Mauthner, *Monatsh. Chem.*, 1913, xxxiv, 883.

An article by Gulewitsch[14] now appeared in which he pointed out that a levorotatory substance may be precipitated with carnosine, which interferes with its crystallization, depresses its melting point, and specific rotation. In this article the physical constants of pure carnosine and its nitrate are recorded. Von Fürth and Hryntschak[15] next attempted to determine carnosine colorimetrically. The intensity of the blue color of a solution of the copper compound or that of the red of the diazo compound, was estimated. The average value found by these methods was 0.27 gm. of carnosine per 100 gm. of muscle. These authors also attempted to determine quantitatively carnosine as the copper salt. In most cases the values were considerably lower than those obtained by the colorimetric methods. In one instance, however, they isolated 0.26 gm. and found 0.298 gm. by the colorimetric method. As to the carnosine content of other organs little is known. Buglia and Costantino,[11] by estimating the nitrogen content of the silver fraction, found 0.045 per cent of carnosine nitrogen in heart and 0.036 per cent in smooth muscle. Von Winiwarter[12] employed the same method and obtained 0.045 per cent for human and 0.026 per cent for equine uterine muscle. Suzuki, Joshimura, Jamakawa, and Irie[16] found as much as 0.2 gm. of carnosine in 100 gm. of fresh fish muscle. Finally, Drummond[17] isolated carnosine from chicken sarcoma.

DISCUSSION.

The chief object of the present investigation was to determine the constitution of carnosine. Either one of two formulas was possible; viz.,



[14] Gulewitsch, *Z. physiol. Chem.*, 1913, lxxxvii, 1.

[15] von Fürth, O., and Hryntschak, T., *Biochem. Z.*, 1914, lxiv, 172.

[16] Suzuki, U., Joshimura, K., Jamakawa, M., and Irie, Y., *Z. physiol. Chem.*, 1909, lxii, 1.

[17] Drummond, J. C., *Biochem. J.*, 1917, xi, 246.

Two ways of solving the problem seemed evident. One, the more conclusive, was to synthesize the base; the other, to prepare deaminocarnosine, hydrolyze it, and then isolate one of its cleavage products.

For the synthesis of carnosine relatively large quantities of histidine were needed. The amino-acid was obtained from dried blood (Armour) by a modification of Fränkel's[18] method.

At first an attempt was made to prepare natural carnosine by the method of Smorodinzew,[7] but this procedure did not lead to the desired result. Only syrupy products were obtained. After several trials, we resorted to precipitation with mercuric acetate after preliminary purification with copper acetate and milk of lime. By this method the extract from several hundred pounds of horse muscle was precipitated. The product thus obtained was purified by the silver process.

Before proceeding to prepare deaminocarnosine, it was advisable to determine the amino nitrogen (Van Slyke) of carnosine and β-alanine. In both cases the analyses agreed well with the calculated amounts for one atom of free amino nitrogen.

Deaminocarnosine was prepared by treating the base with barium nitrite and sulfuric acid. After almost complete deamination had occurred (only 2 per cent of the free amino nitrogen remained), the product was hydrolyzed with acid, without attempting to isolate it. A 70 per cent yield of histidine was obtained, thus indicating that β-alanyl-histidine properly expressed the mode of linkage in the carnosine molecule.

Fischer and Cone[19] had prepared leucyl-histidine by the interaction of bromoisocapronyl chloride and histidine methyl ester, followed by hydrolysis of the resulting ester and amination of the bromo acid. The procedure which was adopted in the present investigation was more convenient. The acid chloride was combined with free histidine in aqueous solution in the presence of an excess of barium hydroxide. The barium was easily removed and the soluble reaction product was aminated without attempting to isolate it. At first we prepared β-iodopropionyl-α-alanine by this method, then β-chloropropionyl chloride was combined with histidine and the reaction product aminated by heating with

[18] Fränkel, S., *Monatsh. Chem.*, 1904, xxiv, 229.

[19] Fischer, E., and Cone, L. H., *Ann. Chem.*, 1908, ccclxiii, 107.

ammonia in a sealed tube. A few crystals were obtained which had the characteristic appearance and optical property of carnosine but the amount was too small for analysis. However, when the product resulting from the reaction of β-iodopropionyl chloride with histidine, was aminated, a substance was formed with properties, optical rotation, and analyses identical with those of natural carnosine.

In order to obtain some preliminary knowledge concerning the fate of carnosine in the body, a study of its behavior toward organ ferments was made. The extracts of dog liver and muscle failed to hydrolyze carnosine. The activity of these extracts had been demonstrated on another dipeptide, glycyl-tryptophane.

The preparation of histidyl-β-alanine or its derivatives was also attempted. Fischer and Cone[19] had tried to prepare histidyl chloride but failed. Their attempt to prepare the chloride of formyl-histidine led to no definite result. We attempted to prepare the chloride of benzoyl-histidine which was easily obtained in a 75 per cent yield by the method of Pauly,[20] but without success. Pauly[21] has also described the preparation of di-β-n phthalene-sulfonyl-histidine. Our attempts to prepare this compound were unsuccessful. α-Naphthalene-sulfonyl chloride, purified by distillation in high vacuum (0.3 mm.) was then combined with histidine according to Fischer and Bergell.[22] However, the properties and analysis of the resulting substance indicated that it was a salt of naphthalene-sulfonyl-histidine with naphthalene sulfonic acid and not the diacyl compound. Naphthalene-sulfonyl-histidine was easily prepared from this salt. Our endeavors to make the chloride of this compound were entirely futile. In this connection it is interesting to note that Pyman[23] failed to prepare the chloride of glyoxaline carboxylic acid, though a number of methods were tried.

Another attempt to synthesize a derivative of histidyl-β-alanine may be mentioned. The main reactions involved in the well known Erlenmeyer[24] synthesis for amino-acids may be expressed as follows:

[20] Pauly, H., *Ber. chem. Ges.*, 1910, xliii, 2243.
[21] Pauly, *Z. physiol. Chem.*, 1904, xlii, 508.
[22] Fischer, E., and Bergell, P., *Ber. chem. Ges.*, 1902, xxxv, 3779.
[23] Pyman, F. L., *J. Chem. Soc.*, 1916, cix, 186.
[24] Erlenmeyer, E., Jr., *Ann. Chem.*, 1893, cclxxv, 1, 8, 13.

$$C_6H_5CONHCH_2COOH + RCHO \rightarrow C_6H_5CONHC(:CHR)COOH \xrightarrow{H_2} C_6H_5CONHCH(CH_2R)COOH$$

By analogy, hippuryl-β-alanine might react with glyoxaline aldehyde[23] thus leading to the benzoyl derivative of histidyl-β-alanine.

$$C_6H_5CONHCH_2CONHCH_2CH_2COOH + C_3H_3N_2CHO \rightarrow C_6H_5CONHC(:CHC_3H_3N_2)CONHCH_2CH_2COOH \xrightarrow{H_2} C_6H_5CONHCH(CH_2C_3H_3N_2)CONHCH_2CH_2COOH$$

We accordingly coupled hippuryl chloride with β-alanine and obtained a beautiful crystalline product which was purified without difficulty. Our attempts to condense it with the easily accessible benzaldehyde were unsuccessful. We abandoned the project at this point.

All our efforts, therefore, to prepare histidyl derivatives of amino-acids have been without avail.

EXPERIMENTAL.

Preparation of Histidine.

Histidine was prepared from dried blood essentially as described by Steudel.[25] The method was altered by the use of litharge to make the removal of hydrochloric acid more complete than could be accomplished by distillation alone.

Considerable quantities of *l*-leucine were obtained by evaporating the mother liquor of the histidine mercury compound, after removal of the metal with hydrogen sulfide.

Preparation of Carnosine.

Fresh hashed horse muscle was extracted in 10 pound portions by covering with tap water and gradually heating to the boiling point. The coagulum was separated by filtration through a cotton bag, and washed with boiling water. About 75 liters of water were used for 100 pounds of meat. The extract was evaporated in shallow pans to about 15 liters, filtered, and treated with 1 kilo of copper acetate and enough milk of lime to make the solution strongly alkaline to litmus. The voluminous precipitate was filtered and thoroughly washed. The filtrate was slightly acidified with sulfuric acid and precipitated with mercuric acetate and

[25] Steudel, H., in Abderhalden, E., *Handb. Biochem. Arbeitsmethoden*, Berlin and Vienna, 1910, ii, 505.

sodium carbonate. The reaction was kept slightly acid. The mercury compound was washed, suspended in water, and decomposed with hydrogen sulfide. The solution was freed of hydrogen sulfide by aeration and treated with a small excess of silver nitrate. Barium hydroxide was added until the reaction was distinctly alkaline to litmus. After a few hours the precipitate was removed and the filtrate saturated with barium hydroxide. The carnosine silver was thoroughly washed with dilute barium hydroxide solution and decomposed with hydrogen sulfide. After removal of the barium with carbon dioxide and sulfuric acid the solution was neutralized with nitric acid and concentrated. About 50 gm. of crude carnosine nitrate were obtained from 100 pounds of horse muscle. The free base was prepared from the nitrate by precipitation with phosphotungstic acid or preferably, by precipitation with silver nitrate and barium hydroxide. The carnosine thus obtained melted at 254° (uncorrected). Its properties were similar to those described by Gulewitsch. It analyzed as follows:

0.0548 gm. substance required 9.6 cc. 0.1 N sulfuric acid.
0.0215 " " gave 0.00148 gm. N according to Van Slyke (micro method).

	Calculated for carnosine $C_9H_{14}O_3N_4$:	Found:
Total nitrogen	24.77	24.52
Amino "	6.2	6.8

Preparation and Hydrolysis of Deaminocarnosine.

4 gm. of carnosine dissolved in 75 cc. of water were deaminated by the addition of 3.2 gm. of barium nitrite and 20 cc. of 0.1 N sulfuric acid. After 5 hours an additional 6.5 cc. of the acid were added. The solution was placed in the refrigerator until the next morning. After heating on the water bath and aerating, it was filtered and diluted to 200 cc. 2 cc. of the solution gave 0.095 cc. of nitrogen gas at 743.5 mm. pressure and 25°, when analyzed according to Van Slyke for amino nitrogen. This was equivalent to but 2.08 per cent of the amino nitrogen originally present. The solution was hydrolyzed by boiling 4 hours with 5 N sulfuric acid. The acid was then removed with barium hydroxide, and the filtrate evaporated in vacuum, and then treated with alcohol. 1.66 gm. of flaky crystals were obtained, which analyzed as follows:

0.0868 gm. substance required 16.5 cc. 0.1 N sulfuric acid (Kjeldahl).

	Calculated for histidine $C_6H_9O_2N_3$:	Found:
N	27.08	26.61

The substance was converted into its hydrochloride, which melted at 255°. Pure histidine hydrochloride melts at the same temperature. When both compounds were mixed there was no change in the melting point. Another portion of the substance was converted into its dihydrochloride by evaporating in a desiccator over sodium hydroxide with concentrated hydrochloric acid. The melting point of the dihydrochloride was 244–245°, that of pure histidine dihydrochloride was 245°. Mixing both compounds did not depress the melting point.

0.0801 gm. substance required 10.6 cc. 0.1 N sulfuric acid (Kjeldahl).

	Calculated for histidine dihydrochloride $C_6H_{11}O_2N_3Cl_2$:	Found:
N	18.4	18.5

The total quantity of histidine obtained from deaminocarnosine was 70 per cent of the theoretical.

β-Iodopropionyl-Alanine ($ICH_2CH_2CONHCH(CH_3)COOH$).

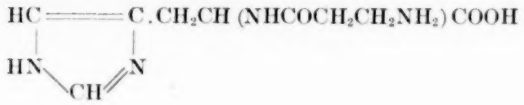
To a solution of 10 gm. of α-alanine dissolved in 300 cc. of water 15 gm. of crystalline barium hydroxide were added. The mixture was cooled with a freezing mixture and agitated with a mechanical stirrer. 21.3 gm. of β-iodopropionyl chloride diluted with petrolic ether were added in the course of 1 hour. In order to maintain an alkaline reaction, an additional 28 gm. of barium hydroxide were required. The excess of barium was removed with carbon dioxide. The chlorine and the remainder of the barium were removed with silver sulfate and sulfuric acid, respectively. After concentration of the solution 18.5 gm. of the reaction product were obtained. The melting point was 153–155°. The substance was dried in vacuum over sulfuric acid.

0.3420 gm. substance required 12.8 cc. 0.1 N sulfuric acid (Kjeldahl).

	Calculated for β-iodopropionyl-alanine $C_6H_{10}O_3NI$:	Found:
N	5.17	5.27

The compound was more soluble in alcohol and acetone than in water, soluble with difficulty in chloroform, and almost insoluble in benzene and petrolic ether.

Synthesis of Carnosine.



40 gm. of free histidine were dissolved in 500 cc. of water and cooled with a freezing mixture. 59 gm. of β-iodopropionyl chloride,[26] diluted to 150 cc. with petrolic ether, were added in the course of 1½ hours. Crystalline barium hydroxide, 140 gm. in all, was added at intervals to maintain alkalinity. Stirring was continued for 2½ hours. The barium was quantitatively removed with carbon dioxide and ammonia. The filtrate was cooled with a freezing mixture and saturated with ammonia. After 48 hours at 37°, the solution was evaporated to 150 cc. and again saturated with ammonia in the cold. Further amination was carried out in sealed tubes at 100°. Owing to breakage of the glass tubes the contents of two were lost. The remainder was evaporated to dryness in vacuum at 45°. The residue was dissolved in water and fractionally precipitated with silver nitrate and barium hydroxide. The precipitate which first appeared, when the solution was made distinctly alkaline, was gummy in nature and care was taken to separate this from the carnosine fraction itself. The precipitate which appeared upon complete saturation with barium hydroxide was washed with dilute baryta solution, decomposed with sulfuric acid, treated with hydrogen sulfide, and filtered. The last trace of sulfuric acid was removed and the solution concentrated. The crystals which soon appeared resembled carnosine in every detail. The yield was about 3 gm. After two recrystallizations the substance melted at 250–251° (corrected). Crystallization was carried out by dissolving in the least amount of warm water and adding pure absolute alcohol. (As carnosine is a strong base it must not be exposed to an atmosphere containing carbon dioxide for too long a time.)

[26] Jacobs, W. A., and Heidelberger, M., *J. Biol. Chem.*, 1915, xxi, 467.

0.0866 gm. substance gave 0.1511 gm. carbon dioxide and 0.0538 gm. water.

0.1222 gm. substance required 21.3 cc. 0.1 N sulfuric acid (Kjeldahl).
0.1044 " " " 18.25 " 0.1 N " " "

	Calculated for carnosine $C_9H_{14}O_3N_4$:	Found:	
C	47.78	47.59	
H	6.19	6.21	
N	24.77	24.40	24.47

The specific rotation of the substance in aqueous solution was + 21.5°, which corresponds with the value recorded for pure natural carnosine.

$$[\alpha]_D^{20} = \frac{4.5550 \times 2.68°}{0.5490 \times 1.034} = +21.5°$$

Action of Liver and Muscle Extracts on Carnosine.

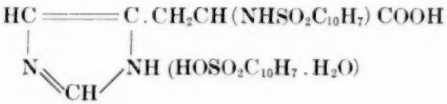
A dog was bled from the carotid artery under ether anesthesia and perfused with 0.9 per cent sodium chloride solution until most of the blood had been removed from the organs. Portions of the liver and muscle were ground with clean sea-sand and extracted with the saline solution. Both extracts hydrolyzed glycyltryptophane after 24 hours at 37°. A solution of carnosine containing 10.18 mg. of amino nitrogen was neutralized with acetic acid and diluted to 10 cc. with Henderson's phosphate solution. To 4 cc. of this solution, 1 cc. of the organ extract and 1 cc. of toluene were added. The control determinations were prepared in a similar manner without the carnosine. After 24 hours at 40°, the contents of the tubes were acidified with dilute acetic acid, coagulated by heat, filtered, washed, and diluted to 10 cc. Amino nitrogen (Van Slyke) was determined in 2 cc. portions of the solution. The temperature of the room was 27° and the barometric pressure 742.5 mm.

	Amino nitrogen. cc.	mg.
(*a*) Liver extract	0.55	0.295
(*b*) " " plus carnosine	2.03	1.088
(*c*) Muscle "	0.27	0.145
(*d*) " " plus carnosine	1.80	0.965

The amino nitrogen added to the extracts, in the form of carnosine, was 0.814 mg. Deducting *a* from *b* and *c* from *d* 0.793 mg.

and 0.820 mg., respectively, are obtained from the liver and muscle mixtures. Hydrolysis did not occur in either case. Abderhalden and Fodor[27] have shown that β-alanine is deaminated in alkaline solution. To see if the slightly alkaline Henderson's phosphate solution decomposed β-alanine, the following experiment was carried out. A quantity of β-alanine equivalent to 1.2490 mg. of amino nitrogen per cc. was dissolved in Henderson's phosphate solution and incubated for 24 hours at 40°. At the end of the period 1.269 mg. were found; hence the alkalinity of the phosphate solution is not sufficient to decompose the amino-acid.

α-Naphthalene-Sulfonyl-Histidine-Naphthalene-Sulfonate.



To a solution of 1 gm. of histidine hydrochloride in 10 cc. of water, 5 cc. of 10 per cent sodium hydroxide and 3 gm. of α-naphthalene-sulfonyl-chloride, dissolved in ether, were added. This mixture was shaken on the machine for 4 hours. During this time 9 cc. of 10 per cent alkali were added in three equal portions. The ether was now removed and the liquid extracted with fresh portions of ether three times. After adding 10 cc. of 5 N hydrochloric acid, an oil appeared which crystallized at room temperature but not in the refrigerator. The crystals are best described as hexagonal plates. The melting point was 146°. Repeated crystallization from water raised the melting point to 155°. On further heating, the fused substance solidified but melted again at 220°. The weight of the substance remained constant when heated over boiling toluene in vacuum for 6 hours in the presence of phosphorus pentoxide.

0.1237 gm. substance gave 0.0975 gm. barium sulfate (sodium peroxide fusion method).

0.1227 gm. substance required 6.8 cc. 0.1 N sulfuric acid (Kjeldahl).

0.1227 " " were neutralized by 4.35 cc. 0.1 N sodium hydroxide solution. Calculated, 4.30 cc.

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[27] Abderhalden, E., and Fodor, A., *Z. physiol. Chem.*, 1913, lxxxv, 112.

0.3167 gm. substance gave 0.6359 gm. carbon dioxide and 0.1338 gm. water.

	Calculated for $C_{20}H_{22}O_8N_4S_2$:	Found:
C	54.6	54.7
H	4.4	4.7
N	7.35	7.76
S	11.2	10.8

In order to remove the crystal water it was necessary to heat the substance to 150° over phosphorus pentoxide in a vacuum. 0.5114 gm. lost 0.0191 gm. of water and became darker. Calculated for one molecule of crystal water, 3.1 per cent. Found, 3.7 per cent. The discrepancy was accounted for by the slight decomposition.

The substance was also prepared by adding naphthalene-sulfonic acid to naphthalene-sulfonyl-histidine. The crystal form of the compound prepared in this manner was similar to that already described. The melting point and solubilities also indicated that both compounds were identical. Owing to difficulties of purification and analysis we were unable to obtain acceptable analytical figures.

0.2085 gm. substance gave 0.4200 gm. carbon dioxide and 0.0900 gm. water.

	Calculated for $C_{20}H_{22}O_8N_4S_2$:	Found:
C	54.6	55.45
H	4.4	4.8

The substance was soluble, with difficulty, in water; more easily soluble in alcohol. When dissolved in boiling methyl alcohol containing a few drops of glacial acetic acid and cooled, long prismatic rods appeared. The melting point was 222° (uncorrected). The substance was dried by heating in a vacuum over boiling toluene for 6 hours in the presence of phosphorus pentoxide.

0.2089 gm. substance gave 0.4339 gm. carbon dioxide and 0.0809 gm. water.

0.1038 gm. substance gave 0.0870 gm. barium sulfate (sodium peroxide fusion method).

0.1100 gm. substance required 4.00 cc. 0.1 N sodium hydroxide solution for neutralization. Calculated, 3.97 cc.

0.1812 gm. substance required 10.10 cc. 0.1 N sulfuric acid (Kjeldahl).

	Calculated for $C_{36}H_{32}O_7N_4S_2$:	Found:
C	56.42	56.64
H	4.19	4.30
N	7.60	7.76
S	11.57	11.51

The analytical data indicated that we were dealing with the anhydrous form of the compound already described.

α-Naphthalene-Sulfonyl-Histidine.

The alcoholic solution of the preceding compound was treated with an amount of 0.1 N sodium hydroxide slightly in excess of neutrality. The solution was evaporated to dryness after the addition of acetic acid and the residue crystallized from methyl alcohol. A further crop of crystals was obtained by diluting the mother liquor with water. Recrystallization was effected by dissolving in dilute ammonia and precipitating with dilute acetic acid. The pure compound appeared in the form of hexagonal blocks, melting at 236° (uncorrected.) The substance lost 5.4 per cent of its weight when heated over boiling toluene in vacuum in the presence of phosphorus pentoxide. Calculated for one molecule of crystal water, 4.98 per cent.

0.2097 gm. substance gave 0.4296 gm. carbon dioxide and 0.0825 gm. water.

0.1225 gm. substance required 10.65 cc. 0.1 N sulfuric acid (Kjeldahl).

0.1082 " " " 3.1 " 0.1 N sodium hydroxide for neutralization. Calculated, 3.13 cc.

0.1082 gm. substance gave 0.0732 gm. barium sulfate (sodium peroxide fusion method).

	Calculated for naphthalene-sulfonyl-histidine $C_{18}H_{15}O_4N_3S$:	Found:
C	55.61	55.87
H	4.47	4.38
N	12.17	12.17
S	9.27	9.29

The substance was very slightly soluble in water, more so in alcohol. It readily dissolved in dilute ammonia or in alcohol containing naphthalene-sulfonic acid. The specific rotation of the sodium salt was determined in aqueous solution.

$$[\alpha]_D^{21} = \frac{3.6852 \times -6.72^\circ}{1.020 \times 0.1272} = -190.8^\circ$$

The rotation of the salt of naphthalene-sulfonic acid with naphthalene-sulfonyl-histidine was also determined. An amount of the substance corresponding to 0.0841 gm. of the sodium salt of naphthalene-sulfonyl-histidine was dissolved in the dimolecular equivalent of sodium hydroxide solution.

$$[\alpha]_D^{24} = \frac{4.8228 \times -3.39^\circ}{1.02 \times 0.0841} = -190.8^\circ$$

The presence of sodium naphthalene sulfonate did not influence the optical activity of the histidine compound. At first we hoped that naphthalene-sulfonyl-histidine would be well adapted for the isolation and characterization of histidine, but this does not seem likely as the yield was only 50 per cent, when pure histidine was used.

Hippuryl-β-Alanine.

3 gm. of β-alanine dissolved in an equivalent of sodium hydroxide solution were combined with hippuryl chloride in the usual manner. 42 cc. of sodium hydroxide (N solution) were added in the course of 30 minutes. Upon acidifying with 9 cc. of N hydrochloric acid, long tyrosine-like needles appeared. The yield was 50 per cent. The melting point was 183–185° (uncorrected). The substance was dried at 100° in vacuum over phosphorus pentoxide.

0.1028 gm. substance required 4.2 cc. 0.1 N sodium hydroxide for neutralization. Calculated, 4.1 cc.

0.1028 gm. substance required 8.5 cc. 0.1 N sulfuric acid (Kjeldahl).

	Calculated for hippuryl-β-alanine $C_{12}H_{14}O_4N_2$:	Found:
N	11.20	11.57

The substance is soluble with difficulty in water and ethyl acetate, but readily in alcohol. It is more soluble in cold water than hippuric acid.

SUMMARY.

The constitution of carnosine has been determined in two ways: (1) By the hydrolysis of deaminocarnosine and isolation of histidine; (2) by the synthesis of carnosine.

The behavior of carnosine toward liver and muscle has been determined.

Attempts to synthesize histidyl-β-alanine were unsuccessful.

AN OXIDATION PRODUCT OF CREATINE.*

By LOUIS BAUMANN and THORSTEN INGVALDSEN.

(From the Chemical Research Laboratory, Department of Internal Medicine, Iowa State University, Iowa City.)

(Received for publication, June 13, 1918.)

After the removal of carnosine from the muscle extract[1] an abundant separation of a glistening flaky material occurred. Investigation showed that this substance consisted of mercury, acetic acid, and an insoluble crystalline compound. The properties and analysis of this compound indicated that it was α-methylguanido-glyoxylic acid,[2] a result of the oxidation of creatine by mercuric acetate.

Many years ago Dessaignes[3] showed that creatine was oxidized to methylguanidine oxalate when heated with mercuric oxide in watery solution. More recently Dakin[4] obtained glyoxylic acid when creatine was treated with hydrogen peroxide and ferrous ulfate. The various stages of creatine oxidation may therefore be represented as follows:

$$NH_2C(:NH)N(CH_3)CH_2COOH \xrightarrow{+O_2} NH_2C(:NH)N(CH_3)CHOHCOOH \xrightarrow{+O_2}$$

$$NH_2C(:NH)N(CH_3)COCOOH \xrightarrow{+H_2O} NH_2C(:NH)N(CH_3)H + COOHCOOH.$$

α-methylguanido-glyoxylic acid is also formed when pure creatine or creatinine is dissolved in mercuric acetate solution and kept at room temperature for several days. Even glycocyamine and glycocoll are oxidized under similar conditions.

* The data are taken from a dissertation submitted by Thorsten Ingvaldsen to the Graduate Faculty of the State University of Iowa, as a partial requirement for the degree of Doctor of Philosophy.

[1] Baumann, L., and Ingvaldsen, T., *J. Biol. Chem.*, 1918, xxxv, 263.

[2] We are indebted to Mr. Carleton E. Curran, of the editorial staff of *Chemical Abstracts*, for suggesting this term.

[3] Dessaignes, M., *Compt. rend. Acad.*, 1854, xxxviii, 839.

[4] Dakin, H. D., *J. Biol. Chem.*, 1905–06, i, 271.

In the well known Engeland[5] process for the separation of nitrogenous muscle extractives, precipitation with mercuric chloride and sodium acetate is carried out. After several days the precipitate is heated with dilute hydrochloric acid and decomposed with hydrogen sulfide. Under these conditions the possibility of creatine oxidation and liberation of methylguanidine must be considered.

TABLE I.

Effect of Injection of α-Methylguanido-Glyoxylic Acid on the Composition of the Urine.

Day.	Volume.	Creatinine.	Creatine.*	Total nitrogen.	Urea nitrogen.	Ammonia nitrogen.
	cc.	*mg.*	*mg.*	*gm.*	*gm.*	*gm.*
1	400	172	16	3.547	2.942	0.210
2	410	165	15	3.659	3.201	0.186
3†						
4†	630	172	30	3.144	2.646	0.106
5†	425	180	144	3.258	2.673	0.183
6	410	174	17	2.727		
7	545	183	3			
8	470	174	4			
11†	400	177	3			
12†	400	168	5			
13†	435	180	9			
14†	545	180	6			
15†	620	172	17			

* Expressed as creatinine.

† From 0.1750 to 0.4000 gm. of α-methylguanido-glyoxylic acid were injected each day.

EXPERIMENTAL.

Preparation of α-Methylguanido-Glyoxylic Acid.

$$NH_2C(:NH)N(CH_3)COCOOH\,.\,2\,H_2O.$$

20 pounds of hashed beef muscle were extracted in small quantities by suspending in water and gradually heating to boiling. Aluminum hydroxide was added as a clearing agent. The clear watery filtrate was concentrated to about 4 liters, and enough

[5] Engeland, R., *Z. Untersuch. Nahrungs- u. Genussmittel*, 1908, xvi, 658.

silver acetate and barium hydroxide were added to remove chlorine and phosphorus, respectively. The filtrate was precipitated with mercuric acetate. A neutral or slightly acid reaction was maintained with the aid of sodium carbonate solution. After 2 hours the precipitate was removed and the filtrate permitted to remain at room temperature for 3 days. During this time copious precipitation had occurred. The solid was removed, washed, suspended in water, and decomposed with hydrogen sulfide. After removing mercuric sulfide and aerating, crystals appeared in the filtrate. An additional amount was obtained by extracting the mercuric sulfide with hot water. In all 10 gm. of substance were obtained from 20 pounds of beef muscle. The crystals appeared as glistening flakes and melted at 203–204° (uncorrected).

The air-dried substance analyzed as follows:

0.3210 gm. substance lost 0.0640 gm. water when heated at 100° in vacuum over phosphorus pentoxide.

0.1070 gm. substance required 17.7 cc. 0.1 N sulfuric acid (Kjeldahl).

	Calculated for $C_4H_2O_3N_2.2H_2O$:	Found:
H_2O	19.89	19.94
N	23.20	23.16

0.0663 gm. substance (dried over phosphorus pentoxide at 100° in vacuum for 6 hours) required 13.6 cc. 0.1 N sulfuric (Kjeldahl).

	Calculated for $C_4H_2O_3N_2$:	Found:
N	28.96	28.72

0.1440 gm. substance was boiled for 1 hour with 100 cc. of 4 per cent crystalline sodium acetate solution. Calcium chloride and a little acetic acid were now added and the boiling was continued for a short time. The precipitated calcium oxalate was filtered, dissolved in dilute hydrochloric acid, and reprecipitated. Found, 0.0454 gm. of calcium oxide; calculated, 0.0445 gm.

The compound is very insoluble in cold, more soluble in hot water. It reacts acid to litmus, but dissolves in dilute mineral acids as well as alkalies. Though it does not react with calcium chloride in the cold, heating precipitates the oxalate in a very short time. It gives off all its nitrogen on prolonged boiling with barium hydroxide solution.

The new compound is somewhat toxic. 0.1500 gm. injected into a guinea pig, weighing 350 gm. produced tonic contractions, stu-

por, and death within 2 hours. Injection into a dog increased the elimination of creatine in one experiment.[6] The weight of the bitch was 7 kilos, and its diet consisted of dried bread and milk. The urine was obtained by a catheter.

[6] The last two experiments were performed by Mr. H. M. Hines to whom we wish to express our thanks.

REDUCTION OF ALDEHYDES TO CORRESPONDING ALCOHOLS.

I. REDUCTION OF HEPTYLIC ALDEHYDE (OENANTHOL).

By P. A. LEVENE and F. A. TAYLOR.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, June 26, 1918.)

Two methods are in vogue for the reduction of aldehydes into alcohols. In one the reduction is accomplished by means of zinc dust and is carried out in a solution of glacial acetic acid. The second consists in the use of sodium amalgam. The disadvantage of the first method lies in the time required for the completion of the reaction, and in the fact that the alcohol formed by this process combines with the acetic acid and is isolated in form of the corresponding acetate.

The reduction with sodium amalgam was first carried out in an aqueous solution. This condition was found unsatisfactory because of the low degree of solubility of the higher aldehydes in it. The process was subsequently modified and glacial acetic acid was employed as solvent. Through this modification the yield of the alcohol was somewhat improved. However, part of the alcohol formed an ester with the acetic acid. The yield was approximately 40 per cent of the theory.

Very recently the reduction of aldehyde to alcohol was accomplished through the process of hydrogenation, by means of hydrogen in the presence of colloidal platinum. This method from the standpoint of yield was very satisfactory. It is, however, troublesome when the reduction of large quantities of aldehyde is desired.

Because some imperfection is attached to every one of the existing methods an attempt was made to reduce aldehydes directly by means of metallic sodium. The process was essentially the same as employed in this laboratory for the reduction of esters to the corresponding alcohols. A fine emulsion of the metal

in toluene was made and glacial acetic acid was used as a solvent for the aldehyde.

The apparatus for the reduction was the same as described by Levene and Allen for the reduction of esters. The details of the operation are described below. Here it suffices to mention that the reaction is completed within 1 hour and that the alcohol remains uncombined with the acetic acid. The yield is about 60 per cent, which is higher than the one obtained either by means of zinc dust or by sodium amalgam.

The method will be extended to the reduction of other aldehydes both of the aliphatic and of the aromatic series.

EXPERIMENTAL.

N-Heptylic Alcohol.—Cenanthol, which boiled between 152 and 156°, was reduced in 30 gm. lots in the apparatus described by Levene and Allen for the reduction of esters with sodium and alcohol. Four equivalents of sodium were suspended in 100 cc. of dry toluene, and the mixture was heated. When the sodium was melted, the stirrer was rotated vigorously in order to form a fine emulsion. The bath was then removed and the flask quickly cooled in ice, the stirring being continued.

The oenanthol, mixed with 50 cc. of glacial acetic acid and 50 cc. of toluene, was then introduced through a dropping funnel at such a rate that there was no refluxing. The mixture was continuously cooled and stirred. In order to complete the reduction and dissolve all the sodium, 50 to 75 cc. of glacial acetic acid were next introduced in the same manner. The whole operation required about an hour.

When the reduction was finished, the flask was removed and treated with water to dissolve the sodium acetate, and the layers were separated. The aqueous solution was saved for treating the next reduction product as, at times, the reaction product contained some undissolved sodium. This solution could be used safely instead of alcohol for completing the solution of the excess sodium.

The toluene layers were united and distilled with steam. The first distillate, mainly toluene and acetic acid, was collected separately. The distillation was continued as long as the odor

of heptylic alcohol persisted. The toluene distillate was fractionated and gave 15 per cent of the total yield of heptylic alcohol.

The oily residue at the end of the distillation gave no further alcohol or acid after saponification with potassium hydroxide.

The combined aqueous solutions obtained from the reductions contained no heptylic alcohol; therefore they were discarded.

The yield of heptylic alcohol from 360 gm. of oenanthol was 208 gm., or 58 per cent of the theoretical. The substance boiled at 173–177°C. On redistillation, the boiling point was 175.5–176.5°C. (corrected).

$$d_4^{25} = 0.8254, \quad n_D^{20} = 1.42463$$

0.1010 gm. substance gave 0.2686 gm. CO_2 and 0.1240 gm. H_2O.

	Calculated for $C_7H_{16}O$: *per cent*	Found: *per cent*
C	72.41	72.52
H	13.77	13.74

N-Heptyl Phenylurethane.—3 gm. of heptyl alcohol and 3 gm. of phenylisocyanate were heated at 175°C. for a few minutes, cooled, and the solid product was recrystallized from dilute alcohol. After four recrystallizations the melting point was constant at 60°C. (corrected). It crystallizes in needles.

0.2000 gm. substance neutralized 8.49 cc. 0.1 N HCl (Kjeldahl).

	Calculated for $C_{14}H_{21}O_2N$: *per cent*	Found: *per cent*
N	5.95	5.94

CEPHALIN V.

HYDROCEPHALIN OF THE EGG YOLK.

By P. A. LEVENE and C. J. WEST.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, June 28, 1918.)

Thudichum[1] who discovered cephalin represented its composition in the following manner:

$$O=P \begin{cases} C_{17}H_{28}O_3 \text{ (cephalyl)} \\ C_{18}H_{35}O_2 \text{ (stearyl)} \\ C_3H_7O_3 \text{ (glyceryl)} \\ C_5H_{11}ON \text{ (neuryl)} \end{cases} C_{43}H_{81}NPO_9$$

The various samples of cephalin analyzed by him had an elementary composition which differed from that required by theory.

	Calculated:	Found:
C	65.59	60.00
H	10.38	9.39
N	1.78	1.68
P	3.94	4.27

The composition of the substance led to an empirical formula of $C_{42}H_{69}NPO_{13} + 5H_2O$. Analyzing the discrepancy of the theoretical and empirical composition of the substance Thudichum writes:

"But the assumption of the presence of five molecules of water of hydration brings the constituent hydrogen to so low a figure that it cannot be explained out of the sum of the chemolytic products. On the other hand the assumption of the presence of five atoms of loosely attached oxygen meets also with theoretical objections. The difficulty here touched requires evidently experimental elucidation."

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[1] Thudichum, J. L. W., A treatise on the chemical constitution of the brain, based throughout on original researches, London, 1884, 57, 58.

Subsequent workers have modified the view of Thudichum on the nature of two of the components of cephalin. Parnas[2] has demonstrated that cephalinic acid is an unsaturated normal octadecylic acid, and assumed it to belong to the diolefin series. Baumann[3] and Renall[4] have shown that the basic substance of cephalin has the structure of amino ethyl alcohol. With this modification the theory requires the following composition of cephalin.

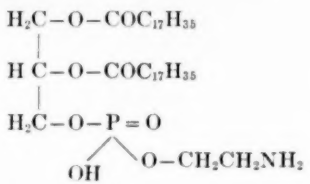
C	H	N	P
per cent	*per cent*	*per cent*	*per cent*
66.16	10.57	1.88	4.18

This composition differs from the empirical even more than the one required by Thudichum's theory. None of the subsequent workers on cephalin have made any serious effort to explain the discrepancy. Our own[5] efforts to prepare a sample of cephalin or a derivative of it, having the composition required by theory, were not very successful.

Yet it is important to bear in mind that nearly all samples of cephalin described by various workers possessed practically identical compositions. Hence, one faces the following dilemma: Either cephalin as generally prepared contains a component which up till the present has escaped identification, or it contains a constant impurity. Recently Levene and West[6] have shown that dehydrolecithin, having a composition required by theory, still contained about 20 per cent of its nitrogen in the form of amino nitrogen. In this fact they saw an indication of the possibility of isolating cephalin having the theoretical composition. This expectation has now been realized. From material prepared in the manner described in the previous article, which was a mixture of reduced lecithin and cephalin, hydrocephalin has been obtained which possessed the composition required by theory, and which had in one sample 93 per cent of its total nitrogen in form of amino nitrogen and in another 97 per cent.

[2] Parnas, J., *Biochem. Z.*, 1909, xxii, 411.
[3] Baumann, A., *Biochem. Z.*, 1913, liv, 30.
[4] Renall, M. H., *Biochem. Z.*, 1913, lv, 296.
[5] Levene, P. A., and West, C. J., *J. Biol. Chem.*, 1916, xxiv, 41; xxv, 517.
[6] Levene and West, *J. Biol. Chem.*, 1918, xxxiv, 175.

Thus the structure of reduced cephalin may be regarded as established and expressed as follows.



The reason for the difficulty in obtaining the non-reduced substance having a theoretical composition still remains obscure.

Assuming cephalinic acid to contain three double bonds and further assuming that each molecule of the substance precipitated in the usual way contains four and a half molecules of water adhering to it, one obtains a calculated composition which does not differ essentially from the empirical.

	C	H	N	P
	per cent	*per cent*	*per cent*	*per cent*
Calculated	60.54	10.54	1.72	3.82
Found (averages)	60.31	9.50	1.80	3.70

Thus far there exists no other experimental evidence for or against this supposition. However, the fact that reduced cephalin of a theoretical composition is obtainable proves that cephalin itself possesses essentially the structure generally assumed for it.

A point in the structure of cephalin which is still under discussion is that of the structure of the glycerophosphoric acid contained in it. Frankel and Dimitz have claimed that the glycerophosphoric acid in cephalin differs from that of lecithin, the latter rotating to the left, the former to the right. It was found that hydrocephalin had an optical rotation in the same direction and practically of the same magnitude as hydrolecithin. In the reduced cephalin as in the reduced lecithin an unsymmetric carbon atom may arise only in the glycerophosphoric acid when the phosphoric acid is attached to one of the terminal carbon atoms. Reduced cephalins and lecithin differ only in that the base in one is amino ethyl alcohol and in the other choline. It is hard to believe that these two substances so closely related structurally and possessing practically the same optical rotation should be derived from two enantiomorphous glycerophosphoric acids.

Attempts to isolate the glycerophosphoric acid from cephalin are now in progress in this laboratory.

EXPERIMENTAL.

The starting material was in part the same which was described by Levene and West in a previous article and in part another lot prepared in the identical manner. The previously described sample contained 20 per cent of its total nitrogen in the form of amino groups. The new material (No. 402) contained 13.09 per cent.

The separation was completed in three phases. The first phase aimed to remove the traces of unsaturated material that might adhere to the substance. This step is accomplished by dissolving the crude material in chloroform and by pouring the solution into a large excess of dry ether. The analysis of the product obtained by this purification (No. 439) is reported in the accompanying table.

The second step accomplished a concentration of the cephalin part so that the mixture consisted approximately of equal parts of the two components. This phase was arrived at through repeated fractional crystallizations out of a solution consisting of equal parts by volume of chloroform and ethylmethyl ketone; 1 part of air-dry material is dissolved in 15 parts of the solvent. As a rule four crystallizations suffice to achieve this phase.

The final purification is accomplished by the recrystallization of the partially purified material out of a solution consisting of 1 part of chloroform and of 2 parts of ethyl alcohol (99.5 per cent); 1 part of the air-dry material is dissolved in 16 parts of the solvent. The crystallization is allowed to proceed at room temperature, and care is taken to prevent evaporation of the chloroform. Out of the mother liquor on standing at about 10°C. a second precipitate is formed which contains about 50 per cent of cephalin. The mother liquor of this material on standing at room temperature in an open flask loses part of its chloroform, and then gives rise to a precipitate which contains only a low proportion (14.86 per cent) of cephalin.

The newly prepared sample will be designated Sample A, and the older one Sample B.

Sample A.

Phase I, No. 402.

0.1034 gm. substance gave 0.2474 gm. CO_2., 0.1034 gm. H_2O, and 0.0108 gm. ash.

0.1938 " substance required for neutralization 3.43 cc. 0.1 N acid.

0.2903 " " gave 0.0424 gm. $Mg_2P_2O_7$.

A sample of the material contained 0.03791 gm. total N and 0.00497 gm. NH_2-N.

Phase II, No. 442.

0.1004 gm. substance gave 0.2422 gm. CO_2 and 0.098 gm. H_2O.

0.1912 " " required for neutralization 2.83 cc. 0.1 N acid.

0.2868 " " gave 0.0410 gm. of $Mg_2P_2O_7$.

A sample of the material contained 0.03842 gm. total N and 0.01133 gm. NH_2-N.

No. 459.

0.0998 gm. substance gave 0.2410 gm. CO_2 and 0.0962 gm. H_2O.

0.1912 " " required for neutralization 2.73 cc. 0.1 N acid.

0.2868 " " gave 0.0414 gm. $Mg_2P_2O_7$.

A sample of the material contained 0.02078 gm. total N and 0.007623 gm. NH_2-N.

No. 463.

0.0956 gm. substance gave 0.2310 gm. CO_2, 0.0938 gm. H_2O, and 0.0094 gm. ash.

0.2000 " " required for neutralization 2.73 cc. 0.1 N acid.

0.3000 " " gave 0.0436 $Mg_2P_2O_7$.

A sample of the material contained 0.0354 gm. total N and 0.01771 gm. NH_2-N.

Phase III, No. 465.

0.1058 gm. substance gave 0.2520 gm. CO_2, 0.0992 gm. H_2O, and 0.0102 gm. ash.

0.1940 " " required for neutralization 2.73 cc. 0.1 N acid.

0.2910 " " gave 0.0448 gm. $Mg_2P_2O_7$.

A sample of the material contained 0.01204 gm. total N and 0.01124 gm. NH_2-N.

Filtrates from No. 465.

No. 547. A sample of the material contained 0.01344 gm. total N and 0.001998 gm. NH_2-N.

$$[\alpha]_D^{20} = \frac{+0.30 \times 100}{0.5 \times 10} = +6.0$$

Sample B.

Phase I was reported in a previous paper.

Phase II, No. 557.

0.1054 gm. substance gave 0.2612 gm. CO_2, 0.0954 gm. H_2O, and 0.1059 gm. ash.

0.1932 " " required for neutralization 2.53 cc. 0.1 N acid.

0.2898 " " gave 0.0393 gm. $Mg_2P_2O_7$.

A sample of the substance gave 0.008064 gm. total N and 0.005255 gm. NH_2-N.

$$[\alpha]_D^{20} = \frac{+0.30 \times 100}{0.5 \times 10} = +6.0$$

Phase III, No. 559.

0.0996 gm. substance gave 0.2386 gm. CO_2, 0.0934 gm. H_2O, and 0.0100 gm. ash.

0.1934 " " required for neutralization 2.67 cc. 0.1 N acid.

0.2901 " " gave 0.0403 gm. $Mg_2P_2O_7$.

A sample of the material gave 0.01383 gm. total N and 0.1361 gm. NH_2-N.

$$[\alpha]_D^{20} \text{ in chloroform} = \frac{+0.30 \times 100}{1 \times 5} = +6.0$$

	Phase of purification.	C	H	N	P	$\frac{NH_2\text{-N}}{\text{total N}}$	Rotation $[\alpha]_D^{20}$
		per cent	*per cent*	*per cent*	*per cent*		
Sample A.	I No. 402	65.25	10.70	2.13	4.07	13.09	
	II_1 " 442	65.79	10.91	2.08	3.98	31.60	
	II_2 " 459	65.86	10.81	2.00	4.02	36.59	
	II_3 " 463	65.89	10.98	1.91	4.05	50.00	
	III " 465	65.0	10.50	1.97	4.29	93.02	
	" 547					14.86	+6.0
Sample B.	I	65.50	11.30	1.80	3.90	20.00	
	II " 556	65.26	10.32	2.01	3.97	65.19	+6.0
	III " 559	65.33	10.50	1.94	3.87	98.33	+6.0
Theory for reduced cephalin, $C_{41}H_{82}O_8NP$.....		65.80	11.05	1.87	4.15	100.00	

FORMATION OF THE UNSATURATED ACIDS IN THE ANIMAL ORGANISM.

First Paper.

THE BEHAVIOR OF *p*-METHOXYPHENYLPROPIONIC ACID IN THE ORGANISM OF THE RABBIT.

By IWAO MATSUO.

(From the Laboratory of Medicine, Imperial University of Kioto, Kioto.)

(Received for publication, June 14, 1918.)

According to the hypothesis of β-oxidation, as established by Knoop,[1] side chains such as $-CH_2CH_2COOH$, $-CH_2CH_2CH_2CH_2$-COOH, etc., attached to a benzene nucleus should be finally oxidized in the animal body to a $-COOH$ group. It seems very probable that these side chains are oxidized, to a $-COOH$ group not directly, but through several intermediary stages. The intermediary products, $RCHOHCH_2COOH$, $R.COCH_2$-COOH, $R.COCH_3$, and $RCH:CHCOOH$ have been isolated.[2]

Dakin[3] found cinnamoylglycocoll in the urine of a cat to which phenylpropionic acid had been administered. Sasaki[4] detected furfuracrylic glycine in the urine of a dog which had been given furfurpropionic acid.

Sasaki isolated 2.7 gm. of furfuracrylic glycine in the urine of a dog after injecting 9 gm. of furfurpropionic acid. Dakin could only isolate 0.45 gm. of cinnamoylglycine from 4 gm. of phenylpropionic acid injected into a cat, and in some cases he could not isolate any cinnamoylglycine.

This remarkable difference in the yields of the two investigators is very interesting. Probably the furan nucleus influences

[1] Knoop, F., *Beitr. chem. Physiol. u. Path.*, 1905, vi, 150.

[2] Dakin, H. D., Oxidations and reductions in the animal body, London, 1912, 17–35.

[3] Dakin, *J. Biol. Chem.*, 1909, vi, 203.

[4] Sasaki, T., *Biochem. Z.*, 1910, xxv, 272.

the oxidation of the side chain differently from the benzene nucleus. Furthermore, different species of animals may produce different results. Dakin stated that he could isolate the cinnamoylglycine from the urine of a cat but not from the urine of a dog.

The writer intends to ascertain the mode of formation of RCH: CHCOOH from RCH_2CH_2COOH in the animal organism by changing both the nucleus and the animal.

In the present experiment *p*-methoxyphenylpropionic acid was selected for the investigation of the behavior of side chain compounds in the animal body. In addition, it is interesting to observe the fate of the methoxy group, for it is a well known fact that guaiacol is converted into catechol in the animal body.

Rabbits were used as experimental animals. The *p*-methoxyphenylpropionic acid was neutralized with sodium carbonate and was injected subcutaneously. Two kinds of crystals were isolated from the urine. Before purification, an alkaline solution of mixed crystals decolorized potassium permanganate. After fractional crystallization, however, neither form of crystals would decolorize permanganate solution. A solution of mixed crystals showed no rotation in the polarimeter. The purified crystals gave neither Millon's reaction nor any color test with ferric chloride. The existence of methoxy groups in both crystalline forms was shown by Zeisel's method.

The two crystalline compounds were identified as anisic acid and the glycocoll derivative of anisic acid.

From the results of my experiments at this time, it appears that unsaturated acids occur as intermediary oxidation products in the animal organisms in only very special cases. This agrees with Dakin's opinion.

Further researches on the formation of unsaturated acids in the animal organism will be continued.

EXPERIMENTAL.

Preparation of p-Methoxyphenylpropionic Acid.

Experiment 1.—According to the direction of Komatsu,[5] tyrosine was methylated with methyl iodide and caustic potash in a

[5] Komatsu, S., *Mem. Coll. Sc., Kioto Imp. Univ.*, 1916, i, 369.

methyl alcoholic solution. The product was *p*-methoxyphenylacrylic acid. It had a melting point of 170–171°.

Experiment 2.—Anisaldehyde and sodium acetate were condensed according to Perkin's method. *p*-Methoxyphenylacrylic acid, melting at 170–171°, was obtained.

p-Methoxypropionic acid was then synthesized by reduction of *p*-methoxyphenylacrylic acid with sodium amalgam and crystallized from hot aqueous solution. It melted at 101–102°.

Feeding Experiments.

After several preliminary tests, the proper dose necessary to avoid too rapid death of the animal was ascertained.

The detailed description of two cases is given.

Experiment 3. Rabbit, ♂, 3,000 Gm.

Date.	*p*-Methoxyphenylpropionic acid injected.	Urine.
1916	*gm.*	*cc.*
Sept. 18	1.5	
" 19	1.5	110
" 20	2.0	85
" 21		70
" 22		Died.
Total	5.0	265

Experiment 3.—The 265 cc. of urine were evaporated to a syrup and extracted with a large amount of hot alcohol repeatedly. The alcohol solutions were evaporated to a residue. This residue was dissolved in a small quantity of water, acidified strongly with 25 per cent phosphoric acid, and extracted twice with hot ether in a Suto-Kumagawa extraction apparatus.

Extraction I (12 Hours).—After removing ether on a water bath, a crude crystalline substance was obtained, which decolorized potassium permanganate immediately. After two crystallizations from hot water, needles melting at 182°C. were obtained. Yield, 0.845 gm. This product would not decolorize potassium permanganate and gave no test for nitrogen. The mother liquor

was concentrated and small flaky crystals melting at 170° separated. The yield was 0.975 gm. This product gave a positive test for nitrogen and did not decolorize potassium permanganate.

Extraction II (24 Hours).—On cooling this ether extract, 0.413 gm. of flaky crystals melting at 169° were obtained. The ether filtrate was evaporated to a syrup. This syrup was removed to a porous plate and allowed to dry and the resulting product was dissolved in hot water. After cooling, 0.348 gm. of needles melting at 181° separated. The mother liquor was concentrated and prisms melting at 170–171° were obtained. The yield was 0.05 gm.

	Melting point.	Crystal form.	Test for nitrogen.	Test with $KMnO_4$.	Yield.
	°C.				gm.
A	181–182	Needles.	−	−	1.193
B	169–171	Flakes or prisms.	+	−	1.438

Experiment 4. Rabbit, ♂, 2,750 Gm.

Date.	*p*-Methoxyphenylpropionic acid injected.	Urine.
1916	gm.	cc.
Sept. 26	1.5	
" 27	1.5	150
" 28	1.5	70
" 29	1.5	140
" 30	2.0	190
Oct. 1	2.0	170
" 2		170
Total	10.0	890

Experiment 4.—In order to avoid bacterial putrefaction the urine was evaporated every 24 hours. The syrups thus obtained were extracted and acidified with phosphoric acid as in the former experiment. It had demonstrated that the compound containing nitrogen was more soluble in water and less soluble in ether than the compound which contained no nitrogen. Therefore, the following procedure was adopted.

I. The acidified solution was extracted with a large amount of cold ether. The ether solution was evaporated to a solid. This solid was then dissolved in hot water and allowed to crystallize. The crystalline material was filtered and again dissolved in hot water. On cooling, white needles separated. The yield was 1.7 gm. The product melted at 182° and gave no test for nitrogen. After concentrating the mother liquor, more needles melting at 181° were obtained. The yield was 0.372 gm. Finally, flakes melting at 170° were obtained. The yield was 0.4 gm. They gave positive test for nitrogen.

II. The remaining phosphoric acid solution was extracted with hot ether for 12 hours. This ether solution was evaporated to dryness and the solid residue was shaken in a separatory funnel with cold ether. The insoluble material was filtered and the filtrate evaporated to dryness. The substance which was soluble in cold ether was crystallized from hot water. Needles melting at 182° were obtained. The yield was 0.677 gm. The material which was insoluble in the cold ether was recrystallized from hot water in the form of prisms melting at 171°. The yield was 0.343 gm.

III. The phosphoric acid solution was again extracted with hot ether for 48 hours. After evaporation and crystallization from water, the only product was prisms melting at 170°. The yield of this extraction was 1.125 gm.

	Melting point.	Crystal form.	Test for nitrogen.	Test with $KMnO_4$.	Yield.
	°C.				gm.
A	181–182	Needles.	−	−	2.749
B	170–171	Prisms or flakes.	+	−	1.868

The product A, melting at 180–182°, was identified as anisic acid.

Analysis.

I. 0.1473 gm. substance gave 0.3376 gm. CO_2 and 0.0697 gm. H_2O.
II. 0.1320 " " " 0.3028 " CO_2 " 0.0624 " H_2O.

	Calculated for $C_8H_8O_3$:	Found: I	II
C	63.15	62.51	62.57
H	5.26	5.26	5.25

Determination of the Methoxy Group by Zeisel's Method.

0.2884 gm. substance gave 0.409 gm. AgI.

	Calculated for $OCH_3C_6H_4COOH$:	Found:
OCH_3	20.39	18.72

The product B, melting at 170–171°, was identified as the glycocoll derivative of anisic acid ($OCH_3C_6H_4CONHCH_2COOH$).

Analysis.

I. 0.1457 gm. substance gave 0.3040 gm. CO_2 and 0.0717 gm. H_2O.
II. 0.2206 " " neutralized 10.7 cc. 0.1 N NaOH solution.
III. 0.1506 " " " 6.9 " 0.1 N NaOH "
IV. 0.2429 " " gave 0.2472 gm. AgI.

	Calculated for $C_{10}H_{11}O_4N$:	Found: I	II	III	IV
C	57.41	56.91			
H	5.26	5.47			
N	6.70		6.80	6.41	
OCH_3	14.83				13.44

The author is indebted to Professor T. Sasaki, of the Department of Medicine, and to Assistant Professor S. Komatsu, of the Department of Organic Chemistry, for their advice throughout this investigation.

THE FOOD VALUE OF EULACHON.*

By MILO REASON DAUGHTERS.

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(Received for publication, June 25, 1918.)

Eulachon is an Indian name given to a species of marine fish found along the Pacific Coast from Oregon to Alaska. Myriads of them come into the fresh water during the first 3 months of the year for the purpose of spawning. The eulachon is related to the smelt and it is often miscalled by this name. Like the salmon, they die after depositing their eggs. The average weight of the 1918 catch at Kelso, Washington, was 52 gm. and their average length 21.5 cm. The edible portion[1] including the roe or milt is approximately 84 per cent.

The results of the chemical examination are recorded in Tables I to III.

TABLE I.

Gross Analysis.

	Edible portion.	As purchased.
	per cent	*per cent*
Moisture	74.66	76.75
Total solids	25.34	23.25
Ash	1.4	1.56
Fat (ether extract)	11.21	9.82
Protein (N × 6.25)	13.18	12.21

* This work was done at the suggestion of Dr. Carl L. Alsberg, Chief of the Bureau of Chemistry, Washington, D. C.

[1] For methods of dressing, cooking, and preserving the eulachon consult *U. S. Bureau of Fisheries, Economic Circ. 33*, 1917.

TABLE II.

Aqueous Extract.

Acidity of extract, 4.65 cc. 0.1 N NaOH per liter.

	per cent
Solids	8.025
Ash	1.2
Organic matter	6.8
Nitrogen compounds.	
Cold water-soluble protein	6.34
Coagulable protein	5.177
Non-coagulable protein	1.16
Proteoses, peptones, gelatin	Trace.
Nitrogen bases	0.571
Amino-acid nitrogen	0.056
Ammonia and amine nitrogen	0.013

TABLE III.

Fat.

Color	Light yellow.	Acid No	0.514
Specific gravity (25°C.)	0.8958	Insoluble acids, *per cent*	94.51
Refractive index (25°C.)	1.4695	Soluble acids, *per cent*	1.49
Melting point, °C	12–25	Hexabromide No	4.75
Iodine No. (Hanus)	122.2	Reichert-Meissl No	1.9
Saponification No	161.5	Titer	24.5
Acetyl No	9.49	Maumené No	59.12

The methods employed are those given in the *Journal of the Association of Official Agricultural Chemists.*[2] Total moisture and solids were determined by drying *in vacuo* over sulfuric acid without heat. Proteoses, peptones, and gelatin were estimated by the tannin-salt method. Sörensen's[3] titration method was used for amino nitrogen, and Folin's aeration method for ammonia and amine nitrogen.

To obtain a sample of fat, the whole fish was passed through a food chopper, placed in a galvanized bucket, and a current of steam introduced near the bottom of the bucket for a period of

[2] Clark, E. D., and Almy, L. H., *J. Assn. Off. Agric. Chem.*, 1916–17, ii, 231. Report of the Committee on Editing Tentative and Official Methods of Analysis, Baltimore, 1916, 271.

[3] Sörensen, S. P. L., *Biochem. Z.*, 1908, vii, 45.

15 minutes, stirring meanwhile. The cooked mass was transferred to a cheese-cloth sack, drained, and then expressed by means of a press. The liquor obtained in this manner, after standing for half an hour, separated into an upper oily and a lower aqueous layer. The lower layer was siphoned off leaving in the container the oil, which was filtered through a dry filter paper and dehydrated by treatment with anhydrous sodium sulfate, from which it was filtered, and then placed in a Freas electric oven at 100°C. for 2 hours. The finished product amounted to more than 300 gm.

Unlike most fish oils, which are liquid at 0°C., the fat of the eulachon is solid at 12°C. and gradually melts until it is all liquid at 25°C.

The hexabromide number was determined by the Bailey-Johnson[4] combination of the methods used by Tolman[5] and Sutcliffe.[6] This method is as follows: About 1 gm. of oil was weighed into a tared weighing bottle 1 inch in diameter by 6 inches long, 25 cc. of absolute ether were added, and the mixture was cooled in ice water. Next there was added slowly drop by drop from a small burette a mixture composed of 5 cc. of bromine and 25 cc. of glacial acetic acid. To produce a deep red color 4 cc. of this brominating agent were necessary. After the addition of the bromine the weighing bottle was allowed to stand in a refrigerator over night. It was centrifuged the next morning for 4 minutes, after which the solvent was decanted from the precipitate, 10 cc. of ice cold absolute ether were added, stirred thoroughly, keeping cold in ice. It was again centrifuged 4 minutes. This process was repeated twice. After the last decantation of ether the container with the hexabromide was dried in an electric oven at 100°C. to constant weight.

The hexabromide from the oil of the eulachon is snow-white in color. It turned brown at 145°C. and melted at 180–184°C.

In food value the eulachon is equal to the salmon. It contains a higher percentage of fat than the salmon and in flavor is considered superior.

[4] Unpublished data. Through the courtesy of Dr. H. S. Bailey in charge of the Oil and Wax Laboratory, Washington.

[5] Tolman, L. M., *J. Ind. and Eng. Chem.*, 1909, i, 340.

[6] Sutcliffe, J. A., *Analyst*, 1914, xxxix, 28.

EFFICIENCY OF MAIZE PROTEIN IN ADULT HUMAN NUTRITION.*

By H. C. SHERMAN and JET C. WINTERS.

(From the Chemical Laboratories of Columbia University, New York.)

(Received for publication, June 22, 1918.)

The proteins of maize were systematically investigated first by Chittenden and Osborne[1] and then by Osborne[2] who found that the maize kernel contains three globulins, a small amount of albumin or proteose, an alcohol-soluble protein which they named zein (reviving the name introduced by Professor Gorham of Harvard University in 1821), and a protein insoluble in water, salt solution, or alcohol, but dissolved by very dilute potassium hydroxide, which has since been designated as maize glutelin. Osborne found in yellow corn-meal, 5.00 per cent of zein, 3.15 per cent of maize glutelin, and 1.48 per cent of all other proteins combined, including the nitrogen compounds insoluble in dilute potassium hydroxide. Later experiments in the same laboratory reported briefly by Osborne and Mendel[3] revealed a much larger proportion of proteins soluble in dilute salt solutions and lead to the conclusion that zein constitutes only two-fifths to one-half of the total protein of the maize kernel, which also is in harmony with the results of Hopkins, Smith, and East.[4]

Zein has been much more extensively investigated than have the other maize proteins. Using their method for the partial separation of the amino-acids resulting from protein hydrolysis, Kossel and Kutscher[5] found no lysine among the hydrolysis products of zein. Osborne and Clapp,[6] using highly developed methods, showed the absence of both lysine and trypto-

* The authors desire to express their indebtedness to the Department of Social Welfare, New York Association for Improving the Condition of the Poor, which shared in the expenses of this investigation by a grant from the research fund established by Mrs. Elizabeth Milbank Anderson.

[1] Chittenden, R. H., and Osborne, T. B., *Am. Chem. J.*, 1891, xiii, 453, 529; 1892, xiv, 20.

[2] Osborne, T. B., *J. Am. Chem. Soc.*, 1897, xix, 525.

[3] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1914, xviii, 1.

[4] Hopkins, C. G., Smith, L. H., and East, E. M., *Illinois Agric. Exp. Station Bull. 87*, 1903.

[5] Kossel, A., and Kutscher, F., *Z. physiol. Chem.*, 1900-01, xxxi, 165.

[6] Osborne, T. B., and Clapp, S. H., *Am. J. Physiol.*, 1907-08, xx, 477.

phane. Willcock and Hopkins[7] showed that a diet in which zein was the sole source of nitrogen was inadequate, and discussed this inadequacy in terms of the absence of tryptophane and lysine. Henriques[8] reported inability of zein to maintain nitrogen equilibrium, and Osborne and Mendel[9] found that with zein as the sole nitrogenous constituent of the diet the experimental animals quickly began to lose both body nitrogen and body weight. But when tryptophane was added to the zein food, maintenance became possible and on the further addition of lysine to the diet growth ensued.[10] McCollum[11] found zein unexpectedly efficient in the replacement of nitrogen lost through endogenous metabolism, the efficiency of zein nitrogen being rated at 80 per cent as against 50 to 60 per cent for gelatin nitrogen. Osborne and Mendel also showed that zein may take the chief part in supplying the material for growth when it is fed in considerable amount along with small amounts of lactalbumin; *e.g.*, with rations containing 13.5 per cent zein and 4.5 per cent lactalbumin, growth was about three times as rapid as with rations containing 4.5 per cent of lactalbumin alone.[12] The superiority of milk proteins to those of maize, or at least of maize endosperm, is well recognized and need not be discussed here, especially as the present paper presents data dealing only with adult maintenance and not with problems of growth or of milk production.

Although Osborne and Clapp[6] showed, in 1908, that maize glutelin contains both lysine and tryptophane, there has too often been manifested a tendency to think of maize protein in terms of zein. This is doubtless one reason for the wide currency given to the estimate of Karl Thomas[13] that the protein of corn-meal is only 30 per cent as efficient in the protein metabolism of maintenance as is meat protein. The evidence offered by Thomas is meager and his methods of experimentation and interpretation appear to have exaggerated greatly the differences in biological efficiency which he was attempting to measure. Osborne and Mendel, in 1914, emphasized the fact that zein constitutes less than half of the protein of the maize kernel and clearly stated, that, "Since maize glutelin, the next most abundant protein, has been shown to yield all of the amino-acids which zein lacks, and as it is probable that the remaining proteins likewise yield them, the amino-acid deficiencies of zein are thus more or less supplemented when the entire seed is fed."[14] In the same paper[15] they show experimentally

[7] Willcock, E. G., and Hopkins, F. G., *J. Physiol.*, 1906–07, xxxv, 88.

[8] Henriques, V., *Z. physiol. Chem.*, 1909, lx, 105.

[9] Osborne and Mendel, *Carnegie Institution of Washington, Publication 156*, 1911.

[10] Osborne and Mendel, *J. Biol. Chem.*, 1914, xvii, 346; 1915, xx, 378.

[11] McCollum, E. V., *Am. J. Physiol.*, 1911–12, xxix, 215.

[12] Osborne and Mendel, *J. Biol. Chem.*, 1914, xvii, 348. Mendel, *J. Am. Med. Assn.*, 1915, lxiv, 1539.

[13] Thomas, K., *Arch. Physiol.*, 1909, 219.

[14] Osborne and Mendel,[3] p. 5.

[15] Osborne and Mendel,[3] p. 12.

that maize glutelin is able to support growth at a normal rate when it constitutes the sole nitrogenous material of the food. On the other hand when the protein of the ration consisted of a mixture of zein and maize glutelin in equal parts, the rate of growth was less than normal.[16,17]

Thus the experiments with isolated proteins fed singly or mixed show that zein alone is inadequate because of its deficiency in lysine and tryptophane, which deficiency may be made good by adding these amino-acids as such to the zein food mixture or by feeding mixtures of zein with proteins which yield lysine and tryptophane on hydrolysis, and that maize glutelin is adequate as a sole protein food but is less efficient as a supplement to zein than is lactalbumin. Since the proteins of maize other than zein and maize glutelin have apparently not been tested separately by feeding experiments, the nutritive efficiency of the mixture of proteins furnished by the corn kernel or its mill products as used for human food cannot be ascertained in a quantitative sense from the data of experiments with isolated proteins. It is of considerable scientific interest to know the efficiency of the natural mixture of proteins furnished by the corn kernel endosperm as compared with that of other seeds. In this country especially, the great quantity of maize produced makes a knowledge of its food value of much economic importance, and particularly at the present time when the mill products of maize are rapidly replacing those of wheat in the nutrition of large numbers of our people. As the result of many and prolonged experiments, chiefly on rats, McCollum[18] has frequently stated that the proteins of wheat and maize are about equally efficient in nutrition. Thomas,[13] from experiments on man, reported low efficiencies for both, with the maize distinctly lower than the wheat; but Hindhede[19] who also worked with human subjects found the protein of wheat bread much more efficient than did Thomas, and has challenged the general validity of the latter's work.

[16] Osborne and Mendel,[3] p. 14.

[17] Further data and discussion on the efficiency of maize protein in growth have been published by Osborne and Mendel in a paper appearing since the above was written (*J. Biol. Chem.*, 1918, xxxiv, 521).

[18] McCollum, *J. Biol. Chem.*, 1914, xix, 329. McCollum, E. V., and Simmonds, N., *ibid.*, 1918, xxxiii, 304.

[19] Hindhede, M., *Skand. Arch. Physiol.*, 1914, xxxi, 259.

In experiments recently reported from this laboratory[20] the substitution of maize protein for that of wheat to the extent of one-fifth to one-third of the total protein intake was found to be without unfavorable effect upon the nitrogen balance, whether the test was made by abrupt substitution in a low protein diet, or by ability to maintain equilibrium for a long time on a low protein diet of which the protein was largely that of corn-meal. In each of these cases there was some discomfort of digestion which was at the time attributed to the unaccustomed use of relatively large quantities of corn-meal, but which is perhaps at least equally attributable to the fact that the diet as a whole was too bulky and too starchy for the summer weather in which the experiments were performed.

In the experiments here reported corn-meal has replaced all other starchy food and has furnished from 73 to 97 per cent of the total nitrogen intake. The subject, a woman of 54 to 55 kilos, not previously accustomed to any large use of corn products, has eaten 300 gm. of corn-meal per day, in two series of experiments each covering 36 days without intermission. The other constituents of the diet were limited amounts of butter fat, sugar, apple, and, in some of the experiments, milk. At no time did the large intake of corn-meal have any detrimental effect upon either appetite or digestion. The corn-meal was prepared in the form of thin hard cakes, known as "corn pones," "scones," or "dodgers" which when eaten were sufficiently hard to ensure thorough mastication. Although the scones were baked at a low oven temperature it is probable that most of the starch reached a higher temperature in the course of its cooking than if it had been prepared as a mush or as muffins or incorporated in ordinary loaves of bread. The starch was so evidently well digested that it was deemed unnecessary to make qualitative or quantitative tests for starch in the feces.[21] The nitrogen of the feces was equivalent to 19 to 32 per cent of the nitrogen intake. Doubtless a considerable part of this was either bacterial or "metabolic" nitrogen or represented

[20] Sherman, H. C., Wheeler, L., and Yates, A. B., *J. Biol. Chem.*, 1918, xxxiv, 383.

[21] Quantitative studies of the action of specific amylases upon wheat, maize, and potato starches are being made in this laboratory in cooperation with the Carnegie Institution of Washington and will be reported elsewhere.

the residues of material expended in digesting the non-nitrogenous constituents of the diet, since the purest preparations of amylase and lipase have been found to be composed essentially of protein material.[22] We have therefore not deducted the nitrogen of the feces from that of the food in our estimation and discussion of the nitrogen intake. If this were done, the apparent efficiency of the maize protein would be very materially increased.

The first experiment lasted 28 days and resulted in a positive nitrogen balance on an intake of 5.85 gm. of nitrogen equivalent to 36.5 gm. of protein of which three-fourths was from corn-meal and one-fourth from milk. The milk was then omitted during the following 8 days which showed an average loss of only 0.63 gm. of nitrogen per day, although the total intake was only 4.26 gm. of nitrogen or 26.6 gm. of protein, corresponding to 34.5 gm. of protein per day for a man of 70 kilos and this entirely corn-meal protein except for the slight amount furnished by the apple. When this second experiment was repeated and continued for 16 days a still closer approach to equilibrium was found, the average daily loss of nitrogen being only 0.46 gm. On introducing 100 gm. of milk into the daily diet (Experiment IV), making the protein intake 30 gm. per day, of which 88 per cent was from maize (corn-meal), 10 per cent from milk, and 2 per cent from apple, a positive nitrogen balance was again obtained.

Thus in the maintenance metabolism of man the efficiency of maize protein as found in these experiments is about the same as that found for wheat protein by Hindhede and (in unpublished experiments) by Sherman and Osterberg. Nitrogen equilibrium was maintained over long experimental periods with a protein intake considerably below that of the Chittenden standard, and of which very much the largest part was maize protein. This would indicate that in as far as the protein requirement of maintenance is concerned one may rely almost entirely upon maize protein even in the case of a very low protein diet. Certainly one need not hesitate on the score of the protein intake to substitute maize products entirely for the corresponding products of wheat, even where these make up the main part of the food supply.

[22] Osborne, T. B., *J. Am. Chem. Soc.*, 1895, xvii, 593. Sherman, H. C., and Schlesinger, M. D., *ibid.*, 1911, xxxiii, 1195; 1912, xxxiv, 1104; 1915, xxxvii, 1305. Sherman, H. C., and Gettler, A. O., *ibid.*, 1913, xxxv, 1790. Falk, K. G., and Sugiura, K., *ibid.*, 1915, xxxvii, 217.

EXPERIMENTAL.

All of the experiments described in this paper were performed upon the same subject, a healthy young woman of 54 to 55 kilos, whose muscular activity consisted in about 8 hours of laboratory work and a walk of about 2 miles daily. Throughout the 72 experimental days covered by the investigation the subject consumed 300 gm. of corn-meal per day. This was taken in the form of thin scones or corn pones, prepared as follows: 300 gm. of corn-meal, 40 gm. of butter fat, and 5 gm. of salt were mixed with 400 to 428 gm. of boiling water. The resulting thick mush was made into 12 small cakes about $\frac{3}{8}$ of an inch thick, which were placed in a Freas electric oven set for a temperature of 190°C., and left for 75 minutes. During the first part of this baking period the oven temperature was somewhat below that indicated because of the opening of the oven to introduce the material, and the absorption of heat in the evaporation of water from it. At the end of the baking the scones were a light golden brown color and crisp almost to the center. Special care was taken to adopt a time and temperature of baking which should involve no loss of nitrogen from the material, as shown by analysis of the finished product of control tests, and yet yield a product sufficiently hard to ensure thorough chewing and insalivation before swallowing. Besides the corn pones, 30 gm. of butter fat, 35 gm. of sugar, and 300 gm. of apple were eaten each day. In Experiment 1 the diet also included 300 gm. of milk; in Experiments 2 and 3, no milk; in Experiment 4, 100 gm., in Experiment 5, 200 gm., of milk per day. Experiments 1 and 2 were continuous, Experiments 3, 4, and 5 were also continuous. Thus the total of 72 experimental days was arranged in two series each of 36 days divided into nine continuous periods of 4 days each. Nitrogen and phosphorus balances were determined throughout Experiments 1 and 2; nitrogen, phosphorus, and calcium balances throughout Experiments 3, 4, and 5.

The data of intake and output are shown in Tables I to VI.

All foods consumed, except the pure sugar and salt and the purified butter fat, were analyzed for nitrogen, phosphorus, and (except in the first two experiments) calcium. Only distilled water was used. Table II shows the extent to which the elements studied were derived from maize, milk, and apple respectively in the different experiments.

TABLE I.

Kinds and Amounts of Food Eaten with Daily Intake of Calories, Protein, Phosphorus, and Calcium.

Kind of food.	Experiment 1. Wt., 54.6 kg.	Experiment 2. Wt., 55.0 kg.	Experiment 3. Wt., 54.4 kg.	Experiment 4. Wt., 54.0 kg.	Experiment 5. Wt., 54.0 kg.
	gm.	*gm.*	*gm.*	*gm.*	*gm.*
Corn-meal	300	300	300	300	300
Milk	300	—	—	100	200
Apple	300	300	300	300	300
Butter fat	70	70	70	70	70
Sugar	35	35	35	35	35
Calories	2,227	2,030	2,030	2,095	2,160
Protein	36.56	26.63	27.25	30.19	31.63
Nitrogen	5.85	4.26	4.36	4.83	5.06
Phosphorus	1.00	0.86	0.46	0.55	0.88
Calcium	.....	.....	0.08	0.23	0.33

TABLE II.

Amounts and Proportions of Nitrogen, Phosphorus, and Calcium from Different Foods in Each Experiment.

Experiment No...	1		2		3		4		5	
	Nitrogen.									
	gm.	*per cent*	*gm.*	*per cent*	*gm.*	*per cent*	*gm.*	*per cent*	*gm.*	*per cent*
Total	5.85		4.26		4.36		4.83		5.06	
Maize	4.29	73	4.14	97	4.23	97	4.23	88	3.99	79
Milk	1.44	25	—	—	—	—	0.47	10	0.94	19
Apple	0.12	2	0.12	3	0.13	3	0.13	2	0.13	2
	Phosphorus.									
Total	1.00		0.86		0.46		0.55		0.89	
Maize	0.68	68	0.82	95	0.42	91	0.42	77	0.67	75
Milk	0.28	28	—	—	—	—	0.09	16	0.18	20
Apple	0.04	4	0.04	5	0.04	9	0.04	7	0.04	5
	Calcium.									
Total					0.08		0.20		0.33	
Maize					0.05	63	0.05	25	0.05	16
Milk					—	—	0.12	60	0.25	75
Apple					0.03	37	0.03	15	0.03	9

TABLE III.

Daily Output of Nitrogen and Phosphorus in Experiments 1 and 2.

Experimental period.	Date.	Body weight.	Nitrogen.			Phosphorus.		
			Urine.	Feces.	Total.	Urine.	Feces.	Total.
Experiment 1.								
	1918	*kg.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Period I.	Feb. 5	53.5	5.65	0.96	6.61	—	—	—
	" 6		5.09	0.96	6.05	0.46	0.49	0.95
	" 7		4.22	0.96	5.18	0.42	0.49	0.91
	" 8		4.50	0.96	5.46	0.37	0.49	0.86
" II.	" 9	55.4	5.09	1.55	6.64	0.44	0.76	1.20
	" 10		4.33	1.55	5.88	0.45	0.76	1.21
	" 11		5.18	1.55	6.73	0.46	0.76	1.22
	" 12		4.26	1.55	5.81	0.41	0.76	1.17
" III.	" 13	55.0	3.83	1.00	5.22*	0.42	0.57	1.02†
	" 14		4.30	1.00	5.69*	0.43	0.57	1.03†
	" 15		4.27	1.00	5.66*	0.43	0.57	1.03†
	" 16		4.31	1.00	5.70*	0.46	0.57	1.06†
" IV.	" 17	54.2	4.79	1.14	5.93	0.45	0.62	1.07
	" 18		3.97	1.14	5.11	0.41	0.62	1.03
	" 19		4.50	1.14	5.64	0.41	0.62	1.03
	" 20		4.52	1.14	5.66	0.39	0.62	1.01
" V.	" 21	54.4	4.68	1.15	5.83	0.43	0.59	1.02
	" 22		4.62	1.15	5.77	0.41	0.59	1.00
	" 23		4.37	1.15	5.52	0.37	0.59	0.96
	" 24		4.72	1.15	5.87	0.43	0.59	1.02
" VI.	" 25	54.4	4.64	1.25	5.89	0.35	0.70	1.05
	" 26		4.25	1.25	5.50	0.35	0.70	1.05
	" 27		4.81	1.25	6.06	0.48	0.70	1.18
	" 28		4.37	1.25	5.62	0.41	0.70	1.11
" VII.	Mar. 1	55.2	4.52	0.95	5.47	0.43	0.50	0.93
	" 2		4.61	0.95	5.56	0.38	0.50	0.88
	" 3		4.55	0.95	5.50	0.42	0.50	0.92
	" 4		3.60	0.95	4.55	0.37	0.50	0.87

* Including 0.39 gm. of nitrogen for each of 4 days from menstrual flow.
† " 0.025 " " phosphorus " " " 4 " " " "

TABLE III—*Concluded.*

Experimental period.	Date.	Body weight.	Nitrogen.			Phosphorus.		
			Urine.	Feces.	Total.	Urine.	Feces.	Total.
Experiment 2.								
	1918	*kg.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Period I	Mar. 5	54.9	3.83	1.42	5.25	0.34	0.71	1.05
	" 6		3.81	1.42	5.23	0.38	0.71	1.09
	" 7		3.84	1.42	5.26	0.40	0.71	1.11
	" 8		3.68	1.42	5.10	0.33	0.71	1.04
" II.	" 9	55.0	3.47	1.32	4.79	0.38	1.05	1.43
	" 10		3.28	1.32	4.60	0.44	1.05	1.49
	" 11		2.88	1.32	4.20	0.33	1.05	1.38
	" 12		3.40	1.32	4.72	0.33	1.05	1.38

TABLE IV.

Daily Output of Nitrogen, Phosphorus, and Calcium in Experiments 3, 4, and 5.

Experimental period.	Date.	Body weight.	Nitrogen.			Phosphorus.			Calcium.		
			Urine.	Feces.	Total.	Urine.	Feces.	Total.	Urine.	Feces.	Total.
Experiment 3.											
	1918	*kg.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Period I.	Mar. 28	54.8	3.95	0.73	4.68	0.31	0.24	0.55	0.05	0.15	0.20
	" 29		4.15	0.73	4.88	0.31	0.24	0.55	0.06	0.15	0.21
	" 30		4.35	0.73	5.08	0.33	0.24	0.57	0.06	0.15	0.21
	" 31		3.81	0.73	4.54	0.31	0.24	0.55	0.06	0.15	0.21
" II.	Apr. 1	54.9	3.78	1.19	4.97	0.21	0.30	0.51	0.05	0.18	0.23
	" 2		3.93	1.19	5.12	0.27	0.30	0.57	0.05	0.18	0.23
	" 3		4.05	1.19	5.24	0.33	0.30	0.63	0.06	0.18	0.24
	" 4		3.30	1.19	4.49	0.30	0.30	0.60	0.05	0.18	0.23

TABLE IV—*Concluded.*

Experimental period.	Date.	Body weight	Nitrogen.			Phosphorus.			Calcium.		
			Urine.	Feces.	Total.	Urine.	Feces.	Total.	Urine.	Feces.	Total.
Experiment 3—*Continued.*											
	1918	*kg.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Period III.	Apr. 5	54.0	3.73	1.20	4.93	0.31	0.29	0.60	0.06	0.18	0.24
	" 6		5.18	1.20	4.03*	0.13	0.29	0.46†	0.05	0.18	0.24‡
	" 7		3.36	1.20	5.21*	0.29	0.29	0.62†	0.06	0.18	0.25‡
	" 8		3.55	1.20	5.40*	0.30	0.29	0.63†	0.07	0.18	0.26‡
" IV.	" 9	53.7	3.71	0.88	5.24*	0.30	0.23	0.57†	0.07	0.15	0.23‡
	" 10		3.33	0.88	4.21	0.24	0.23	0.47	0.05	0.15	0.20
	" 11		3.80	0.88	4.68	0.30	0.23	0.53	0.05	0.15	0.20
	" 12		3.52	0.88	4.40	0.20	0.23	0.43	0.07	0.15	0.22
Experiment 4.											
Period I.	Apr. 13	53.8	3.76	0.89	4.65	0.26	0.27	0.53	0.10	0.19	0.29
	" 14		3.35	0.89	4.24	0.28	0.27	0.55	0.09	0.19	0.28
	" 15		3.88	0.89	4.77	0.29	0.27	0.56	0.10	0.19	0.29
	" 16		3.37	0.89	4.26	0.24	0.27	0.51	0.08	0.19	0.27
" II	" 17	54.1	3.66	1.07	4.73	0.23	0.38	0.61	0.11	0.25	0.36
	" 18		3.75	1.07	4.82	0.18	0.38	0.56	0.07	0.25	0.32
	" 19		3.74	1.07	4.81	0.29	0.38	0.67	0.07	0.25	0.32
	" 20		3.63	1.07	4.70	0.25	0.38	0.63	0.11	0.25	0.36
" III.	" 21	54.2	3.47	1.02	4.49	0.09	0.45	0.54	0.05	0.25	0.30
	" 22		3.94	1.02	4.96	0.19	0.45	0.64	0.06	0.25	0.31
	" 23		4.17	1.02	5.19	0.26	0.45	0.71	0.09	0.25	0.34
	" 24		3.77	1.02	4.79	0.30	0.45	0.75	0.07	0.25	0.32
Experiment 5.											
Period I.	Apr. 25	53.7	3.86	1.33	5.19	0.34	0.58	0.92	0.09	0.36	0.45
	" 26		3.70	1.33	5.03	0.37	0.58	0.95	0.09	0.36	0.45
	" 27		3.65	1.33	4.98	0.34	0.58	0.92	0.10	0.36	0.46
	" 28		3.15	1.33	4.48	0.29	0.58	0.87	0.11	0.36	0.47
" II.	" 29	54.6	4.16	1.34	5.50	0.36	0.60	0.96	0.07	0.34	0.41
	" 30		3.43	1.34	4.77	0.29	0.60	0.89	0.08	0.34	0.42
	May 1		3.53	1.34	4.87	0.28	0.60	0.86	0.08	0.34	0.42
	" 2	53.9	3.33	1.34	4.67	0.34	0.60	0.94	0.09	0.34	0.43

* Including 0.65 gm. of nitrogen for each of 4 days from menstrual flow.
† " 0.043 " " phosphorus " " " 4 " " " "
‡ " 0.013 " " calcium " " " 4 " " " "

TABLE V.

Summary of Daily Intake and Output in Nitrogen and Phosphorus in Experiments 1 and 2.

	Period.	Body weight.	Nitrogen.			Phosphorus.		
			Intake.	Output.	Balance.	Intake.	Output.	Balance.
		kg.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Experiment 1, 4 day periods.	I	53.5	5.85	5.83	+0.02	1.00	0.91*	+0.09
	II	55.4	5.85	6.27	−0.42	1.00	1.21	−0.21
	III	55.0	5.85	5.56	+0.29	1.00	1.03	−0.03
	IV	54.2	5.85	5.59	+0.26	1.00	1.04	−0.04
	V	54.4	5.85	5.74	+0.11	1.00	1.00	±0.00
	VI	54.4	5.85	5.76	+0.09	1.00	1.10	−0.10
	VII	55.2	5.85	5.27	+0.58	1.00	0.90	+0.10
Average.....		54.6	5.85	5.72	+0.13	1.00	1.03	−0.03
Experiment 2, 4 day periods.	I	54.9	4.26	5.21	−0.95	0.86	1.07	−0.21
	II	55.1	4.26	4.57	−0.31	0.86	1.42	−0.56
Average.....		55.0	4.26	4.89	−0.63	0.86	1.25	−0.39

* Average of 3 days only.

TABLE VI.

Summary of Daily Intake and Output in Nitrogen, Phosphorus, and Calcium in Experiments 3, 4, and 5.

	Period.	Body weight.	Nitrogen.			Phosphorus.			Calcium.		
			Intake.	Output.	Balance.	Intake.	Output.	Balance.	Intake.	Output.	Balance.
		kg.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Experiment 3, 4 day periods.	I	54.8	4.36	4.80	−0.44	0.46	0.56	−0.10	0.08	0.21	−0.13
	II	54.9	4.36	4.96	−0.60	0.46	0.58	−0.12	0.08	0.24	−0.16
	III	54.0	4.36	4.89	−0.53	0.46	0.58	−0.12	0.08	0.25	−0.17
	IV	53.7	4.36	4.63	−0.27	0.46	0.50	−0.04	0.08	0.21	−0.13
Average.....		54.4	4.36	4.82	−0.46	0.46	0.56	−0.10	0.08	0.23	−0.15
Experiment 4, 4 day periods.	I	53.8	4.83	4.48	+0.35	0.55	0.56	−0.01	0.20	0.28	−0.08
	II	54.1	4.83	4.77	+0.06	0.55	0.62	−0.07	0.20	0.34	−0.14
	III*	54.2	4.83	4.88	−0.05	0.55	0.63	−0.08	0.20	0.32	−0.12
Average.....		54.0	4.83	4.71	+0.15	0.55	0.60	−0.05	0.20	0.31	−0.11
Experiment 5, 4 day periods.	I	53.7	5.06	4.92	+0.14	0.89	0.92	−0.03	0.33	0.46	−0.13
	II	54.6	5.06	4.95	+0.11	0.89	0.91	−0.02	0.33	0.42	−0.09
Average.....		54.1	5.06	4.94	+0.12	0.89	0.92	−0.03	0.33	0.44	−0.11

* Average of 3 days only.

EFFECT OF HEAT ON THE CITRIC ACID CONTENT OF MILK.

ISOLATION OF CITRIC ACID FROM MILK.*

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The citric acid content of milk and the effect of heat on this acid was investigated with the idea of correlating the results with the alleged production of scurvy or Barlow's disease in infants fed heated milk. Citric acid was discovered as a normal constituent of cow's milk by Soxhlet and Henkel in 1888.[1] It was claimed by Obermaier[2] from his work in 1904, that the citric acid content of milk decreased on boiling; he claimed to have observed in some cases as high a decrease as 31.86 per cent of the total. It is believed by several investigators that the antiscorbutic effect of fruit juices[3] is due to their citric acid content; consequently if citric acid is an antiscorbutic and if there was a decrease in the content of this acid on heating milk, this fact might be used to explain, as often claimed, why heated milk should be more conducive to the production of scurvy than raw milk.

Method of Estimation.

In the estimation of the citric acid in the different samples of milk Beau's[4] modification of that of Denigès was used. In this

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[1] Soxhlet and Henkel, T., *Münch. med. Woch.*, 1888, xxxv, 328.

[2] Obermaier, G., *Arch. Hyg.*, 1904, l, 52.

[3] Funk, C., *J. State Med.*, 1912, xx, 341. Gerstenberger, H. J., *Am. J. Med. Sc.*, 1918, clv, 261.

[4] Beau, *Rev. gèn. lait*, 1903-04, iii, 385.

method the casein in the milk is precipitated by a dilute sulfuric acid solution of mercuric sulfate. The clear serum obtained by this precipitation contains the citric acid and an excess of mercuric sulfate. This serum is then heated and the citric acid oxidized to acetone dicarboxylic acid, $CH_2COOH.CO.CH_2COOH$, by the gradual addition of dilute potassium permanganate solution. The acetone dicarboxylic acid forms a precipitate with the mercury. This precipitate is then filtered from the cold solution. The precipitate, dissolved in hydrochloric acid, is next titrated for the amount of mercury by potassium cyanide and silver nitrate, using potassium iodide as an indicator. From the amount of mercury found the amount of citric acid is determined.

EXPERIMENTAL.

Before starting the experiments on the effect of heat on the citric acid content of milk and to satisfy our credulity as to the existence of this acid in milk we undertook its isolation. From 10 liters of skimmed cow's milk the casein was removed by acidifying with hydrochloric acid. The serum was neutralized partly with sodium hydroxide and partly with lime water to the normal acidity of cow's milk, heated, and the coagulated albumin filtered off. The clear serum was concentrated to 0.1 of its original volume. The flocculent precipitate which separated out at this point was assumed to be chiefly calcium citrate. This precipitate was filtered off and dissolved in dilute nitric acid. From this nitric acid solution the citric acid was precipitated as lead citrate by the addition of lead acetate. The lead citrate was suspended in water and decomposed by hydrogen sulfide. After the lead sulfide had been filtered off the solution was concentrated on the steam bath to a syrupy solution, and then placed in a vacuum desiccator over calcium chloride. In a day or two the liquid was filled with crystals. These were recrystallized from water and washed with ether.

In this manner 5 gm. of crystals were obtained from 10 liters of skimmed milk. We identified these crystals as citric acid in the following manner.

I. The crystals were heated with 6 parts of ammonium hydroxide in a sealed tube at 120–130°C. for 6 hours. The contents

were then poured into a flat dish and exposed to light. A blue color gradually developed and finally changed to green. This is the characteristic Sabanin-Laskowski reaction for citric acid.

II. A water solution of the crystals heated with dilute sulfuric acid solution of mercuric sulfate, with the gradual addition of potassium permanganate solution, gave a white precipitate. This is a positive reaction of Denigès' test for citric acid.

III. The crystals were dried for 48 hours in a vacuum over calcium chloride, weighed, dissolved, and titrated with 0.1 N sodium hydroxide, using phenolphthalein as indicator with the following results.

Weight of material.	0.1 N alkali required.	0.1 N alkali required for $C_6H_8O_7$.
gm.	*cc.*	*cc.*
0.4133	64.26	64.57
0.2122	33.56	33.17

Known citric acid crystals dried and titrated in the same manner gave the following results.

Weight of citric acid.	0.1 N alkali required.	0.1 N alkali required for $C_6H_8O_7$.
gm.	*cc.*	*cc.*
0.3872	61.00	60.5

The close agreement of the actual amount of standard alkali used with the theoretical amount is further proof that we had citric acid. The known and unknown were in good agreement, while the data also show that citric acid will lose its water of crystallization by the treatment given these preparations.

IV. Some of the crystals were dissolved and barium acetate was added to the solution. A white precipitate formed. This precipitate was digested on a steam bath for 1 hour and then filtered and washed with hot water and dried in a desiccator. It was then weighed, dried to constant weight at 160°C., and weighed again. Known citric acid was treated similarly.

	Weight.	Lost.	Water.	Theoretical per cent of water in $Ba_3(C_6H_5O_7)_2.7H_2O$.
	gm.	*gm.*	*per cent*	*per cent*
Preparation from milk....	1.7168	0.2392	13.92	13.75
Known citric acid.....	1.2468	0.1688	13.54	13.75

In samples of the water-free barium citrate from milk and also in the known citric acid the amount of barium was determined.

	Weight of sample.	Weight of $BaSO_4$.	Ba found.	Theoretical per cent of Ba in $Ba_3(C_6H_5O_7)_2$.
	gm.	*gm.*	*per cent*	*per cent*
Preparation from milk....	0.2044	0.1807	52.03	52.16
	0.2144	0.1891	51.91	52.16
	0.2247	0.1975	51.73	52.16
Known citric acid.....	0.2256	0.2002	52.22	52.16
	0.2062	0.1833	52.32	52.16

In both the water of crystallization and barium content the barium salt of the unknown citric acid from milk agreed very closely with the theoretical and with known barium citrate.

These data identify the crystals isolated from milk as citric acid and confirm the earlier work of Soxhlet and Henkel.

In the experiments on the effect of heat, raw milk was compared with milk that had been boiled under a reflux condenser for 20 minutes. The results, given in Column I of Table I, show that there was no decrease in the citric acid content. More severe heat treatment was then tried, the milk being boiled for 1 hour under a reflux condenser. Again, as seen from Column II, no change occurred.

Obermaier[2] calls attention to boiling with access to air, claiming under such conditions a loss of 31.86 per cent of the citric acid. To imitate his conditions of experimentation milk was boiled in an open casserole for 30 minutes, with free access to the air in one case and in another experiment with air bubbled through the boiling milk. In each case the water lost as a result of evaporation

TABLE I.

Effect of Heat on the Citric Acid Content of Milk. Gm. per Liter.

I		II		III		IV		V		VI		VII	
A	B	A	C	A	D	A	E	A	F	A	G	A	H
2.42	2.40	2.43	2.48	2.29	2.29	2.29	2.29	2.48	2.48	2.26	2.23	2.46	2.51
				2.32	2.35	2.32	2.29					2.46	2.55
2.29	2.32							2.37	2.42	1.91	1.92		
2.32	2.35							2.39	2.39	1.97			
2.35	2.36												
2.38	2.35												

A = raw milk with corresponding heated milk opposite it.
B = milk boiled under the reflux condenser for 20 minutes.
C = " " " " " " " 1 hour.
D = " " in open casserole for 30 minutes. Evaporation water restored.
E = milk boiled with air bubbled through for 30 minutes. Evaporation water restored.
F = milk autoclaved at 15 pounds pressure for 20 minutes.
G = " " " 15 " " " 1 hour.
H = " " 20 minutes, then filtered, and the opalescent serum compared with the serum from raw milk.

was restored. The data given in Columns III and IV show that even here no change in the citric acid content of the milk occurred.

To determine the influence of high temperatures several samples of milk were heated, some for 20 minutes and later some for 1 hour, in the autoclave at 15 pounds pressure. The results in Columns V and VI again show no loss of citric acid even under these severe treatments. It has been suggested that the citric acid in milk, which is there as mono- and dicalcium citrate, might be changed to the less soluble tricalcium citrate on heating. To test the plausibility of this statement raw milk and milk which had been autoclaved for 20 minutes were filtered through a compact layer of asbestos until an opalescent serum was obtained. The serum on analysis showed no change in citric acid content as can be seen in Column VII. This result is in accord with the conclusion reached by Splittgerber;[5] *viz.*, that citrates in milk are not converted to the insoluble tricalcium citrate on heating.

[5] Splittgerber, A., *Z. Untersuch. Nahrungs- u. Genussmittel*, 1912, xxiv, 493.

SUMMARY.

1. We have confirmed the findings of Scheibe and Henkel; namely, that cow's milk does contain citric acid. The amount found was approximately 0.2 per cent of the milk, or 2 per cent of the milk solids.

2. Citric acid is not destroyed in the heating of milk even in the autoclave at 15 pounds pressure for 1 hour.

3. The citric acid salts of milk are not changed to an insoluble form on heating.

THE FORMATION OF OXYPHENYLLACTIC ACID IN THE ANIMAL ORGANISM AND ITS RELATION TO TYROSINE CATABOLISM.

By YASHIRO KOTAKE.

With the Cooperation of Zenji Matsuoka.

(From the Medical Chemical Institute of the Medical College, Osaka.)

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INTRODUCTION.

Blendermann has obtained from the urine of a rabbit, fed with a large amount of tyrosine, an acid crystallizing in long needles. It was soluble in ether, gave a strong Millon's reaction, and had the composition $C_9H_{10}O_4$ and the presumable constitution OH $CH_2-CHOH-COOH$. The character of this acid and the statement regarding its formation, however, were not given in detail. Consequently, it seems that some authors did not entirely believe in the constitution of the acid as given by Blendermann. Dakin wrote in his monograph: "Schotten observed the excretion of mandelic acid following administration of phenylaminoacetic acid, while Blendermann believed that *p*-hydroxyphenyl-α-lactic acid was present in the urine of rabbits when they were fed large quantities of tyrosine. The constitution of Blendermann's acid is doubtful however."

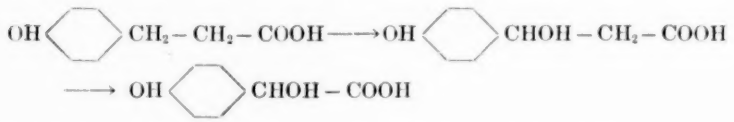
Schultzen and Riess found previously an aromatic acid in the urine of patients who died of acute yellow atrophy of the liver. In spite of the similarity of its properties to those of Blendermann's acid, it had the composition $C_8H_9O_4$ according to its analysis. They believed that it was oxymandelic acid OH $CHOH-COOH$.

After some years an acid having the same properties was again isolated by Baumann from the urine of a man suffering from

phosphorus poisoning, but he could not decide its constitution by analysis because of the small quantity available. Röhmann and von Ackeren also obtained the same acid from the urine in a case of acute yellow atrophy of the liver, without identifying it accurately.

The investigations of Neubauer concerning the fate of oxyphenylpyruvic and oxphenyllactic acid in an alcaptonuric patient, the observations of Kotake and of Suwa upon the cleavage of the same acids in normal human bodies, and the experiments of Embden and Schmitz on the tyrosine synthesis from the above named acids through a surviving liver, all speak in favor of the fact that, of the two acids, only the former is an intermediary stage of the physiological catabolism of tyrosine. Therefore, if Blendermann's acid is really oxyphenyllactic acid, its formation cannot be accepted as exact without further investigations.

On the other hand, according to our present view, it seems difficult to regard oxymandelic acid as a substance formed intermediately in the body. It is well known that putrefying bacteria are able to decompose tryosine yielding thereby oxyphenyllactic acid, so that in special pathological cases a previous formation of the latter and its conversion into oxymandelic acid might be assumed.



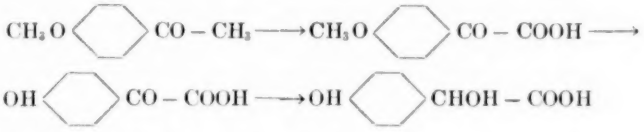
But it is not likely, because oxymandelic acid has hitherto never been found in the urine collected after administering oxyphenyllactic acid. In any case, the constitution of the acid of Schultzen and Riess is very doubtful.

At the suggestion of Dr. Ellinger and in cooperation with him, I attempted to solve this question: Is the acid isolated by Schultzen and Riess true oxymandelic acid?

At first we tried to obtain the acid described by these authors from the urine of a patient suffering from acute atrophy of the liver but were unable to find one. We, therefore, synthesized oxymandelic acid and compared its properties with those of the acid obtained by Schultzen and Riess. After unsuccessful

investigations, we arrived at the synthesis of oxymandelic acid in the following way:

Methoxyacetophenone was successively converted into methoxyphenylglyoxylic, oxyphenylglyoxylic, and oxymandelic acids and finally the latter was split into its optical antipodes:



We found that our synthetic *dl*-, *l*-, and *d*-oxymandelic acids all differed distinctly from the acid of Schultzen and Riess in their melting points. A little later Fromherz also synthesized *dl*- [illegible] and its properties agreed

CORRECTION.

On page 320, Vol. XXXV, No. 2, August, 1918, lines 19 and 27, for *oxyphenyllactic*, read *oxyphenylpropionic*.

Riess and Baumann. It crystallized in long needles melting at 167–168°C. and gave an intensive Millon's reaction. However, this acid, according to its analysis, had the composition $C_9H_{10}O_4$, agreeing with Blendermann's acid. My acid was levorotatory.

On the other hand, the possibility of obtaining a synthetic preparation of *l*-oxyphenyllactic acid was proved, so that by way of comparison, the constitution of my acid was determined with certainty. The synthesis was accomplished by the action of nitrous acid upon *l*-tyrosine, although the yield was not great, and it was found that this synthetic acid was identical in all respects with mine obtained from the urine.

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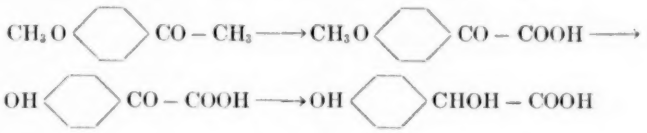

We found that our synthetic *dl*-, *l*-, and *d*-oxymandelic acids all differed distinctly from the acid of Schultzen and Riess in their melting points. A little later Fromherz also synthesized *dl*-oxymandelic acid by another method, and its properties agreed with ours. Subsequently Neubauer carried out the optical investigation of the preparation which was isolated by Schultzen and Riess. It was levorotatory. Thus it appears that the acid isolated by these authors cannot be *l*-oxymandelic acid, since there is a great difference in the melting point (about 60°C.) between this and our *l*-oxymandelic acid.

Following a suggestion by Baumann, I now undertook to obtain from the urine of dogs poisoned with phosphorus an acid which was supposed by Baumann to be identical with the acid of Schultzen and Riess. I obtained a substance that possessed all the properties of the acid described by Schultzen and Riess and Baumann. It crystallized in long needles melting at 167–168°C. and gave an intensive Millon's reaction. However, this acid, according to its analysis, had the composition $C_9H_{10}O_4$, agreeing with Blendermann's acid. My acid was levorotatory.

On the other hand, the possibility of obtaining a synthetic preparation of *l*-oxyphenyllactic acid was proved, so that by way of comparison, the constitution of my acid was determined with certainty. The synthesis was accomplished by the action of nitrous acid upon *l*-tyrosine, although the yield was not great, and it was found that this synthetic acid was identical in all respects with mine obtained from the urine.

Elimination of l-Oxyphenyllactic Acid following the Administration of Tyrosine.

The foregoing facts give fair evidence that the acid of Schultzen and Riess is *l*-oxyphenyllactic acid. Was, therefore, the acid isolated by Blendermann also *l*-oxyphenyllactic acid?

We obtained the following results.

l-Tyrosine, used for our experiment, was obtained by the digestive action of trypsin on fibrin and then completely purified by repeated recrystallizations from hot water. A rabbit weighing 3,150 gm. was used.

Each day the animal was given 10 gm. of tyrosine by means of a stomach tube. Until the 5th day of the experiment, the animal was healthy and its appetite good, but because the animal appeared weak after the 5th day, the feeding of tyrosine was discontinued. The next day a small quantity of protein was detected in the urine.

The daily specimens of urine collected separately during the experiment were evaporated on the water bath to a syrup which was repeatedly extracted by hot alcohol, and the alcoholic extract was carefully evaporated on the water bath. Then the residue was taken up with water, strongly acidified by means of sulfuric acid, and extracted about ten times with ether. After distilling the ether extract, the oily residue was left which, for the most part, crystallized on the next day. It was again dissolved in water and lead acetate was added to it as long as a precipitate formed; then the filtrate was precipitated with basic lead acetate. The precipitate, after washing, was finally suspended in water and decomposed by means of hydrogen sulfide. The filtrate, freed from hydrogen sulfide, was investigated in regard to its rotation and then further evaporated to a syrup. It was allowed to stand for some days in a cool place, causing a crystallization of all the residues obtained on the days on which tyrosine was given. All the results obtained were put together in the following table.

Date.	Urine.	Reaction.	Substance soluble in ether and precipitable by basic lead acetate.	Rotation.	Remarks.
	cc.		*gm.*		
Dec. 22–23	225	Neutral.	Small quantity.	No rotation.	
" 23–24	315	Acid.	0.1958	Levorotatory.	10 gm. tyrosine suspended in 10 cc. water given.
" 24–25	300	"	0.6620	"	" " " "
" 25–26	290	"	0.7984	"	10 gm. tyrosine suspended in 60 cc. water given.
" 26–27	285	"	0.9398	"	" " " " Animal appeared weak.
" 27–28	164	"	0.7540	"	No tyrosine given.
" 28–29	164	"	0.2340	"	Protein detected in the urine. Residue obtained from the ether extract did not crystallize.
" 29–30	180	Slightly acid.	Small quantity.	No rotation.	

All the specimens of substance which were obtained in this way and found optically active, were dissolved together in water, and after again adding some drops of lead acetate solution, a resin-like precipitate formed which was filtered. The filtrate was again precipitated by basic lead acetate and this precipitate was treated with hydrogen sulfide in the same way as before. The solution filtered off from the lead sulfide was evaporated to a small quantity on the water bath and allowed to stand for some days in a cool place. A substance giving Millon's reaction was obtained, which crystallized out in long colorless needles melting at 168°C. It was identified as *l*-oxyphenyllactic acid by the determination of its specific rotation and its water of crystallization and by analysis.

0.5598 gm. substance (dried at 105–110°C.) in 23.5 cc. water, rotated in a 2 dm. tube with D light 0.88° to the left.

$$[\alpha]_D = -18.48^\circ$$

0.1606 gm. substance (dried in air) lost 0.0079 gm. water at 105–110°C.

	Calculated for ($C_9H_{10}O_4 + \frac{1}{2} H_2O$): *per cent*	Found: *per cent*
H_2O	4.71	4.98

0.1481 gm. substance (dried at 105–110°C.) gave 0.3220 gm. CO_2 and 0.0766 gm. H_2O.

	Calculated for $C_9H_{10}O_4$: *per cent*	Found: *per cent*
C	59.34	59.31
H	5.49	5.79

We believe that our reinvestigation furnished evidence enough that the acid of Blendermann was *l*-oxyphenyllactic acid and this result makes it appear probable that *l*-oxyphenyllactic acid may be a normal intermediary product of tyrosine catabolism in the animal body. It must be admitted, however, that this assumption is not likely to be true, if the results obtained by Neubauer, Kotake, and also Suwa, as mentioned above, are taken into consideration. From the results obtained by Blendermann and by us, it can no longer be doubted that *l*-oxyphenyllactic acid appearing in the pathological cases mentioned was also derived from tyrosine. Further investigations of the exact mechanism of tyrosine catabolism are much to be desired.

Formation of l-Oxyphenyllactic Acid from Oxyphenylpyruvic Acid.

Suwa found that a small part of oxyphenylpyruvic acid administered in the human body underwent reduction, so that *d*-oxyphenyllactic acid was formed. Owing to this result, Neubauer advanced the following hypothesis:

"An dem Vorkommen der *l*-Oxyphenylmilchsäure unter verschiedenen pathologischen Bedingungen kann danach nicht gezweifelt werden. Diese Säure kann nur aber nicht als sekundäres Produkt primär gebildeter Ketonsäure angesehen werden; denn wenn Oxyphenylbrenztraubensäure im Organismus (des Menschen) reduziert wird, so entsteht daraus die *d*-Oxyphenylmilchsäure. Somit der Schluss gerechtfertigt, dass *l*-Oxypenylmilchsäure einer primären hydrolytischen NH_3-Abspaltung aus dem Tyrosin ihre Entstehung verdankt, die anscheinend deswegen eintritt, weil der normale oxydative Abbau über die Ketonsäure infolge der Lebererkrankung unmöglich ist."

I found previously, however, that oxyphenylpyruvic acid, n a rabbit, was converted into *l*-oxyphenyllactic acid; although the latter, on account of its small amount, could not be accurately identified. I therefore carried the same experiment further in order to arrive at a conclusion regarding the formation of *l*-oxyphenyllactic acid in the animal body.

For the experiments dogs and rabbits were used, and the result confirmed the one obtained previously; namely, that, contrary to Suwa's result, a small part of the oxyphenylpyruvic acid administered was converted into *l*-oxyphenyllactic acid.

The investigations of Suwa, as mentioned above, were carried out in the human body. I also have performed some experiments in the human body but without obtaining clear results concerning the behavior of oxyphenylpyruvic acid in the animal organism. Recently Matsuoka and I have again taken up this problem. The results obtained were the following.

Experiment 1.—At 8 o'clock, 2 hours after breakfast, one of us, weighing about 50 kilos, and 35 years old, took by mouth 5 gm. of oxyphenylpyruvic acid in the form of the sodium salt. The urine (1,265 cc., specific gravity 1.019), which had been collected during the 24 hours after taking the acid, was evaporated on the water bath nearly to dryness, and repeatedly extracted by hot alcohol until the residue no longer gave Millon's reaction. The alcohol extract was distilled and the residue dissolved in water, strongly acidified by means of dilute sulfuric acid, and extracted several times with ether. After distillation of the ether, the residue was taken up with water, treated with lead acetate, and then with basic lead acetate. The last precipitate was suspended in water, decomposed by hydrogen sulfide, and the solution, filtered from the lead sulfide, was concentrated on the water bath to 18 cc. which gave in a 2 dm. tube a rotation of $-0.14°$. The solution was then, after concentration, allowed to stand in a cool place, and a substance amounting to 0.1 gm., giving Millon's reaction, crystallized out in long needles. We dissolved these crystals melting at 145–155°C. in 14 cc. of water and polarized the solution in a 1 dm. tube. The rotation was $-0.05°$; the specific rotation was therefore $[\alpha]_D = -5.3°$. Since this was less than one-third of that of pure *l*-oxyphenyllactic acid, the solution was again evaporated on the water bath to a small quantity and inoculated with some crystals of *l*-oxyphenyllactic acid. After a short time, 0.04 gm. of crystals, melting at 168°C. and possessing all the properties of *l*-oxyphenyllactic acid, separated out (see analysis below).

From the mother liquor of this *l*-acid, a small quantity of a substance giving Millon's reaction was obtained. Its melting point, after purification, reached 144–145°C. It was optically inactive, so that it is almost certain that we had obtained *dl*-oxyphenyllactic acid.

Thus we arrived at a second problem; namely, where and how *dl*-oxphenyllactic acid is formed in the body. Our attention was drawn to the possibility that the latter acid may be formed in the intestines from oxyphenylpyruvic acid by the action of microorganisms. Consequently we undertook to carry out experiments not only by taking oxyphenylpyruvic acid by mouth but also by injecting it into the human body.

Experiment 2.—At 8.15, 2 hours after breakfast, a man weighing about 60 kilos, and 24 years old, took by mouth 5 gm. of oxyphenylpyruvic acid in the form of the sodium salt. The urine of the next 24 hours (485 cc., specific gravity 1.016) was treated in the same manner as described before. Finally the solution, filtered from the lead sulfide, was evaporated on the water bath to 10.5 cc. and polarized in a 1 dm. tube, showing a rotation of $-0.09°$. From this solution, we isolated 0.02 gm. of a substance possessing all the properties of *l*-oxyphenyllactic acid.

Experiment 3.—At 8.15, 2 hours after breakfast, a man weighing about 50 kilos, and 17 years old, took 6 gm. of oxyphenylpyruvic acid in the form of the sodium salt. The urine of the next 24 hours (1,485 cc., specific gravity 1.016) was treated in the way described above and the solution finally obtained by the decomposition of the precipitate of basic lead acetate was concentrated to 10.5 cc. It showed in a 1 dm. tube a rotation of $-0.13°$. From this solution, we could isolate needle-shaped crystals which melted at 155–160°C. and gave Millon's reaction. They were, however, not a homogeneous compound, so that by fractional crystallization they could be separated into two different substances. One, melting at 222–223°C. could not be identified on account of its small quantity; the other, melting at 144–145°C. and giving Millon's reaction, was optically inactive, and was identified as *dl*-oxyphenyllactic acid by the determination of its water of crystallization and its analysis.

0.1012 gm. substance (dried in the air) lost 0.0048 gm. water at 105–110°C.

	Calculated for ($C_9H_{10}O_4 + \frac{1}{2} H_2O$): *per cent*	Found: *per cent*
H_2O	4.71	4.74

0.0963 gm. substance (dried at 105–110°C.) gave 0.2093 gm. CO_2 and 0.0502 gm. H_2O.

	Calculated for $C_9H_{10}O_4$: *per cent*	Found: *per cent*
C	59.34	59.27
H	5.49	5.84

Experiment 4.—At 11 a.m., before lunch, a man weighing about 70 kilos, and 32 years old, was subcutaneously injected with 7 gm. of oxyphenylpyruvic acid in the form of the sodium salt. In the urine collected

during the next 24 hours we could find neither the *l* nor the *dl* form of oxyphenyllactic acid.

Experiment 5.—At 1.30 p.m., 1½ hours after lunch, one of us was subcutaneously injected with 5 gm. of oxyphenylpyruvic acid which was dissolved in 40 cc. of water and neutralized by sodium carbonate. For some time after the injection, he had a headache and the temperature rose as high as 37.6°C., but the next morning he felt quite well. The urine (1,270 cc., specific gravity 1.020) which had been collected during the 24 hours after the injection, gave a strong Millon's reaction, but protein or bile pigments were not present. The urine was treated in the usual manner and the solution finally obtained by the decomposition of the precipitate of basic lead acetate was concentrated to 10.5 cc. and rotated in a 1 dm. tube, showing −0.26°. On further concentration a substance crystallized out which by its morphology, reaction, melting point (167–168°C.), specific rotation, and analysis was identified as *l*-oxyphenyllactic acid. In the mother liquor *dl*-oxyphenyllactic acid was not present.

0.0670 gm. substance (dried at 105–110°C.) in 10.5 cc. water rotated in a 1 dm. tube with D light −0.12°.

$$[\alpha]_D = -18.81°$$

The analysis of the substance so obtained was carried out after we had added to it the similar substance that we had obtained as a result of Experiment 1.

0.0931 gm. substance (dried at 105–110°C.) gave 0.2022 gm. CO_2 and 0.0490 gm. H_2O.

	Calculated for $C_9H_{10}O_4$: *per cent*	Found: *per cent*
C	59.34	59.23
H	5.49	5.89

These data leave no doubt that oxyphenylpyruvic acid administered by mouth or by injection is, in a small quantity, reduced to *l*-oxyphenyllactic acid and eliminated in the urine. Since oxyphenylpyruvic acid, as mentioned before, may be regarded as a normal product of tyrosine catabolism, it appears reasonable to assume that *l*-oxyphenyllactic acid, found in certain diseased conditions or after administering tyrosine, is formed from the latter, oxyphenylpyruvic acid being an intermediary stage; that is, tyrosine administered or formed promptly and abundantly on account of very active disintegration of tissue proteins due to pathologic process, undergoes, at first, oxidative deamination, yielding oxyphenylpyruvic acid, and the latter is then converted into oxyphenyllactic acid.

In two experiments with oxyphenylpyruvic acid taken by mouth, we could unmistakably detect the presence of *dl*-oxyphenyllactic acid in the urine, whereas we could not find any traces of it in the urine following the injection of oxyphenylpyruvic acid. Thus it appears most probable that the formation of *dl*-oxyphenyllactic acid found by us takes place in the intestinal tract in which such reducing agents as some microorganisms or hydrogen in the nascent state are acting.

The investigations of Aoyama and Yuyen, carried out in our laboratory, showed that *Bacillus coli* is capable of yielding oxyphenyllactic acid at the expense of oxyphenylpyruvic acid, but the acid found by them was also the *l* form, so that this result does not explain the probable formation of *dl*-oxyphenyllactic acid in the intestines.

Although we are not yet able to explain the result of Suwa, that is the elimination of *d*-oxyphenyllactic acid following the administration of oxyphenylpyruvic acid by mouth, we may state here that its formation may take place, secondarily, by the asymmetrical decomposition of *dl*-oxyphenyllactic acid (see below) which was primarily formed in the intestines. In any case it becomes evident that the hypothesis of Neubauer, quoted above, no longer has any basis, because, contrary to the result of Suwa, we could detect *l*-oxyphenyllactic acid in the urine following the administration of oxyphenylpyruvic acid.

Asymmetrical Decomposition of dl-Oxyphenyllactic Acid.

My former experiments with *dl*-oxyphenyllactic acid demonstrate that it is combustible with difficulty in the animal body, because its greater part is eliminated unchanged in the urine. At that time the experiments were carried out only in rabbits, without considering the products which might be formed from the acid in the tissues.

Recently I have repeated the same experiments giving especial attention to the probable asymmetrical decomposition of the *dl*-acid in the animal body. The experiments were carried out on rabbits, dogs, and a man with the following results.

Experiment 6.—A rabbit weighing 2.750 gm., received subcutaneously 2 gm. of *dl*-oxyphenyllactic acid in the form of the sodium salt. The

urine, collected during the 24 hours following, was treated as described above, and a substance isolated from it which was soluble in ether and precipitable by basic lead acetate. The substance had no optical activity.

The substance thus obtained in an amount of about 1.5 gm. was dissolved in water and neutralized with sodium carbonate and again injected subcutaneously into the same rabbit. The substance was isolated from the urine of the next 24 hours by the treatment mentioned above, showing slight rotation to the right.

Experiment 7.—A rabbit weighing 2,350 gm., was subcutaneously injected with 1 gm. of *dl*-oxyphenyllactic acid in the form of the sodium salt. The urine of the 24 hours following the administration of the acid was treated as before. The substance finally obtained showed a dextrorotation.

Two specimens of the substance obtained from Experiments 6 and 7 were united and recrystallized, after having been inoculated with the crystals of *d*-oxyphenyllactic acid. 0.15 gm. of a substance melting at 164–166°C. and giving Millon's reaction was obtained. It was dextrorotatory.

0.1490 gm. substance (dried in air) lost 0.0074 gm. water at 105–110°C.

	Calculated for $(C_9H_{10}O_4 + \frac{1}{2}H_2O)$: *per cent*	Found: *per cent*
H_2O	4.71	4.97

0.1462 gm. substance (dried at 105–110°C.) in 10.5 cc. water rotated in a 1 dm. tube with D light + 0.22°.

$$[\alpha]_D = +14.33°$$

Experiment 8.—A small dog weighing 3,500 gm., received subcutaneously 2 gm. of oxyphenyllactic acid (injected twice at intervals of 2 hours). The urine which had been collected during the 24 hours after the last injection, was treated in the usual manner. The substance finally obtained in an amount of 1 gm. showed a distinct dextrorotation. It was further recrystallized, *d*-oxyphenyllactic acid being used for inoculation, and a substance was separated which gave Millon's reaction. The melting point was 165–167°C.

0.1545 gm. substance (dried in air) lost 0.0073 gm. water at 105–110°C.

	Calculated for $(C_9H_{10}O_4 + \frac{1}{2}H_2O)$: *per cent*	Found: *per cent*
H_2O	4.71	4.72

0.1515 gm. substance (dried at 105–110°C.) in 10.2 cc. water rotated in a 1 dm. tube with D light +0.25°.

$$[\alpha]_D = +16.17°$$

From the foregoing data it is evident that *dl*-oxyphenyllactic acid undergoes asymmetrical decomposition in the body of the

rabbit and the dog, the *d*-acid thereby remaining unattacked. The specific rotations of two specimens obtained from two rabbits and a dog were too slight for the pure *d*-acid. However, this may be regarded as due to the admixture of the *dl*-acid administered.

0.1802 gm. substance (taken from the mixture of two specimens obtained from the rabbit and dog and dried at 105–110°C.) gave 0.3918 gm. CO_2 and 0.0922 gm. H_2O.

	Calculated for $C_9H_{10}O_4$: *per cent*	Found: *per cent*
C	59.34	59.30
H	5.49	5.73

Experiment 9.—A man weighing about 50 kilos, and 35 years old, took by mouth 5 gm. of *dl*-oxyphenyllactic acid in the form of the sodium salt. The result obtained was similar to that of Suwa who showed in experimenting on himself that the *dl*-acid is asymmetrically decomposed and the *d*-acid appears in the urine. The *d*-acid which was isolated by us and purified by fractional recrystallization showed a specific rotation of $[\alpha]_D = +14.01$.

As previously stated, Neubauer found that oxyphenyllactic acid does not yield homogentisic acid when given to an alcaptonuric patient. The acid used by him was, however, the *dl*-acid. From our results it becomes evident that the *l*-acid is more or less readily combustible in the body. I intend, therefore, to repeat the experiment of Neubauer with the *l*-acid once more, in order to obtain a clear picture of the course of the decomposition of *l*-acid in the animal organism.

CONCLUSIONS.

1. The acid found by Blendermann in the urine following the administration of tyrosine is *l*-oxyphenyllactic acid.[1]

2. Oxyphenylpyruvic acid administered in the animal organism is, for the most part, completely decomposed and a small part is converted into *l*-oxyphenyllactic acid, which in its turn is eliminated in the urine. According to our present view, there is no doubt that the above named ketonic acid is the normal intermediary product of tyrosine catabolism. Consequently *l*-oxyphenyllactic acid found after administering tyrosine or in certain

[1] Dr. Matsuoka succeeded in isolating oxyphenylpyruvic acid from the urine of a rabbit fed with tyrosine (not yet published).

pathological conditions, may be regarded as derived from tyrosine (administered or formed in the body) with the intermediary stage of oxyphenylpyruvic acid.

3. *dl*-Oxyphenyllactic acid administered in the animal organism undergoes, in part, asymmetrical decomposition, the *d*-acid remaining unattacked. Thus it becomes evident that the formation of *l*-oxyphenyllactic acid from oxyphenylpyruvic acid in the body takes place by asymmetrical reduction of the latter.

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THE RELATION BETWEEN NUTRITION AND THE FORMATION OF KYNURENIC ACID FROM TRYPTOPHANE.

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The investigations of Abderhalden, Hopkins, Osborne and Mendel, and others give evidence that tryptophane is one of the amino-acids essential for nutrition of the animal body. Nevertheless our knowledge concerning the intermediary stages of its catabolism is not great.

Kynurenic acid, discovered by Liebig in the urine of dogs, was investigated in all details by Ellinger, and it became evident that this acid is formed from tryptophane in the body. But the physiological significance of its transformation was not determined.

In the course of the other experimental investigations, concerning kynurenic acid, I found that the quantity of the latter, following the administration of tryptophane, was not constant, although the same rabbits weighing 2 to 3 kilos were given 1 gm. of tryptophane subcutaneously in each instance. It seems likely that this variation, aside from the individual differences in the animals, was due to the state of their nutrition.

I have therefore carried out some experiments in which the rabbits used were all fed in exactly the same way, and have found in all cases that the elimination of kynurenic acid became almost constant, especially in the same individual. The experiments were then continued to include a study of the exact relation between nutrition and the transformation of kynurenic acid from tryptophane in the animal body.

EXPERIMENTAL.

Rabbits of 2 to 3 kilos were used. Tryptophane for the injection was obtained by the pancreatic digestion of fibrin, and it

was completely purified by recrystallization. Kynurenic acid eliminated in the urine was estimated as follows:

The urine collected during the 24 hours following every administration of tryptophane (or kynurenic acid) was evaporated on the water bath almost to dryness and the residue repeatedly extracted by alcohol until the alcohol was no longer colored. The residue obtained by the evaporation of the alcohol was dissolved in water and acidified with sulfuric acid, whereupon the larger part of kynurenic acid separated out. The mixture was allowed to stand in a cool place, after it had been covered with a small quantity of ether. The next day the precipitate was filtered and washed with water until a portion of the filtrate no longer reacted acid. It was then washed with alcohol and ether, dried at 105°C., and weighed. The mother liquor of kynurenic acid was repeatedly extracted with ether, in order to remove the substances soluble in the latter, and then precipitated with phosphotungstic acid, for the isolation of such substances as kynurenic acid and tryptophane. The precipitate obtained in a small quantity, was treated with baryta water. The filtrate, after the removal of barium, was concentrated and allowed to stand in a cool place, but yielded nothing.

Experiment 1.

During the first period the animal was fed with okara, residue obtained from bean curds, and a small amount of fresh vegetables and several times injected subcutaneously with 1 gm. of tryptophane in the form of sodium salt. The animal was then fasted, but during this second period the sodium salt of tryptophane was repeatedly injected. During a third period the animal was again fed in the same way as during the first period and injected several times with tryptophane. In the course of the experiment the rabbit was occasionally injected with 0.5 gm. of kynurenic acid in the form of sodium salt for the examination of cleavage in the body.

Experiment 2.

The conditions under which the experiment was carried out were similar to those of Experiment 1. The influence of venesection on the formation of kynurenic acid was investigated.

Experiment 3.

The experiment was carried out under the same conditions as Experiment 1. But here the frequency of the administration of tryptophane was reduced in order to avoid as much as possible the stimulation caused by the injection. At the end of the experiment the fate of kynurenic acid was investigated.

CONCLUSIONS.

1. The rate of the formation of kynurenic acid from tryptophane (1 gm. injected) is almost constant in the rabbit, especially in the same individual, if it is fed under the same conditions.

2. This rate is not changed during the actual period of fasting but it is distinctly diminished during the next feeding period, at the time of the recovering from fasting.

3. The influence of venesection on the rate of the formation of kynurenic acid is not ascertainable.

4. In the animal body kynurenic acid is very stable, or its decomposition occurs only to a small extent.

From the results described above it may be inferred that the formation of kynurenic acid from tryptophane does not indicate a phase in the process of nutrition. This consideration is in accord with the result obtained by Asayama; namely, that kynurenic acid cannot take the place of tryptophane for the maintenance of nutrition.

TABLE I.

Date.	Body weight.	Substance injected.	Kynurenic acid.	Urine.	Reaction.	Remarks.
	gm.	*gm.*	*gm.*	*cc.*		
June 24	2,450					
" 30	2,350	1.0 tryptophane.		220	Alkaline.	
July 1	2,380		0.2086	190	Acid.	
" 8	2,430				Alkaline.	
" 9					"	
" 10	2,420				"	
" 11	2,370	1.0 tryptophane.		205	"	
" 12	2,370		0.2248	138	Acid.	
" 13	2,350			160	Alkaline.	
" 14	2,360	1.0 tryptophane.			"	

TABLE I—*Concluded*

Date.	Body weight.	Substance injected.	Kynurenic acid.	Urine.	Reaction.	Remarks.
	gm.	*gm.*	*gm.*	*cc.*		
July 15	2,380		0.2540	176	Slightly acid.	
" 16	2,370	1.0 tryptophane.		244	Alkaline.	
" 17	2,380		0.2428	230	"	
" 18	2,380	1.0 tryptophane.		185	"	
" 19	2,360		0.2292	150	"	
" 25	2,380	1.0 tryptophane.			"	
" 26	2,380		0.2306	156	Acid.	Fasted.
" 27	2,270	1.0 tryptophane.		62	"	"
" 28	2,220		0.2432	53	"	"
" 29	2,160			37	"	"
" 30	2,130	1.0 tryptophane.		38	"	"
" 31	2,080		0.2534	42	"	
Aug. 1	2,020			42	"	
" 2	1,960	0.5 kynurenic acid.		40	"	
" 3	1,930		0.3890	35	"	
" 4	1,890			33	"	Fed.
" 5	2,040				Alkaline.	
" 6	2,220	1.0 tryptophane.		110	"	
" 7	2,310		0.1472	247	"	
" 10	2,320	1.0 tryptophane.		328	"	
" 11	2,340		0.0840	326	"	
" 12	2,340			345	"	
" 13	2,310	0.5 kynurenic acid.	0.3596	245	"	
" 18	2,340	1.0 tryptophane.		198	"	
" 19	2,370		0.2216	205	"	
" 22	2,400	1.0 tryptophane.		135	"	
" 23	2,380		0.2920	167	"	
" 27	2,400	0.5 kynurenic acid.		230	"	Kynurenic acid given by mouth.
" 28	2,400		0.0596	260	"	
Sept. 16	2,460			212	"	
" 17	2,440	1.0 tryptophane.		186	"	
" 18	2,420		0.2150	236	"	
" 19	2,420			170	"	
" 20	2,430			164	"	

TABLE II.

Date.	Body weight.	Substance injected.	Kynurenic acid.	Urine.	Reaction.	Remarks.
	gm.	*gm.*	*gm.*	*cc.*		
June 24	2,700				Alkaline.	
" 30	2,890	1.0 tryptophane.		370	"	
July 1	2,780		0.2422	300	Acid.	
" 11	2,830	1.0 tryptophane.		270	Alkaline.	
" 12	2,850		0.2628	160	Acid.	
" 13	2,840			220	Alkaline.	
" 14	2,820	1.0 tryptophane.			"	
" 15	2,750		0.2236	130	"	
" 16	2,800	1.0 tryptophane.		254	"	
" 17	2,810		0.2706	220	"	
" 18	2,820	1.0 tryptophane.		200	"	
" 19	2,830		0.2630	240	"	
" 25	2,900	1.0 tryptophane.		270	"	
" 26	2,900		0.2190	215	"	
" 27	2,900	1.0 tryptophane.		200	"	25 cc. of blood venesected.
" 28	2,750		0.2440	80	Acid.	
" 29	2,740			183	Alkaline.	
" 30	2,780	1.0 tryptophane.		232	"	
" 31	2,790		0.2402	170	"	
Aug. 1	2,830			225	"	
" 2	2,860	1.0 tryptophane.	0.2280		"	
" 10	2,650	1.0 tryptophane.		84	Acid.	Fasted.
" 11	2,600		0.2273	48	"	"
" 13	2,440	1.0 tryptophane.		33	"	Small quantity of food given by mistake.
" 14	2,440		0.2078	40	"	Fasted Aug. 14–20.
" 19	2,120	1.0 tryptophane.		42	"	
" 20	2,090		0.2246	44	"	
" 21	2,020			36	"	Fed for remainder of experiment.
" 22	2,140	1.0 tryptophane.		55	Alkaline.	
" 23	2,340		0.1682	75	"	
" 26	2,620	1.0 tryptophane.		322	"	
" 27	2,610		0.1268	228	"	
" 31	2,780	1.0 tryptophane.		236	"	

TABLE II—*Concluded.*

Date.	Body weight.	Substance injected.	Kynurenic acid.	Urine.	Reaction.	Remarks.
	gm.	*gm.*	*gm.*	*cc.*		
Sept. 1	2,720		0.0684	248	Alkaline.	
" 6	2,680	1.0 tryptophane.		218	"	
" 7	2,710		0.1472	210	"	
" 11	2,780	1.0 tryptophane.		330	"	
" 12	2,850		0.2238	344	"	
" 17	2,820	1.0 tryptophane.		280	"	
" 18	2,860		0.2234	294	"	
" 27	2,880	1.0 tryptophane.		350	"	
" 28	2,900		0.2228	326	"	

TABLE III.

Date.	Body weight.	Substance injected.	Kynurenic acid.	Urine.	Reaction.	Remarks.
	gm.	*gm.*	*gm.*	*cc.*		
Apr. 25	2,220	1.0 tryptophane.		245	Alkaline.	
" 26	2,230		0.3755	210	Acid.	Fasted Apr. 25–May 5.
" 27	2,195			238	Alkaline.	
May 4	1,800	1.0 tryptophane.		44	Acid.	
" 5	1,740		0.3462	35	"	
" 6	1,690			36	"	Fed for remainder of experiment.
" 13	2,160	1.0 tryptophane.		240	Alkaline.	
" 14	2,130		0.2497	260	"	
" 21	2,305	1.0 tryptophane.		260	"	
" 22	2,260		0.1276	210	Slightly acid.	
" 23	2,290			275	Alkaline.	
" 24	2,300			240	"	
" 28	2,360	1.0 tryptophane.		220	"	
" 29	2,330		0.3548	196	"	
June 4	2,300	1.0 tryptophane.		110	"	
" 5	2,320		0.3602	177	"	
" 12	2,385	1.0 tryptophane.		300	"	
" 13	2,380		0.3356	164	"	
" 19	2,390	0.5 kynurenic acid.		236	"	
" 20	2,375		0.4907	260	"	

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THE DECOMPOSITION OF MUCONIC AND ADIPIC ACIDS IN THE ANIMAL BODY.

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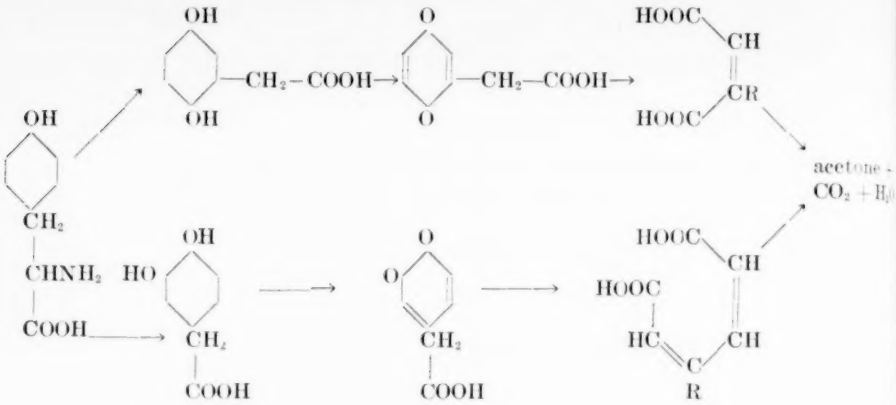
Some aromatic substances readily undergo combustion in the animal organism with the splitting of the benzene ring. It is well known that aromatic amino-acids derived from protein hydrolysis, tyrosine and phenylalanine, are completely oxidized in the body. Most investigators believe that these amino-acids are decomposed over homogentisic acid. In fact the latter is easily burned in the animal body and capable of yielding acetone bodies in the perfusion experiment with a surviving liver. However, the manner in which homogentisic acid is converted into acetone bodies is unknown.

Jaffé discovered an unsaturated acid, namely, muconic acid, in the urine of dogs and rabbits to which benzene had been given, and by that fact demonstrated for the first time the demolition of the benzene nucleus.

At that time Jaffé found that his acid was readily combustible in the animal body, so that when injected into a rabbit to the amount of 2 gm., only 1 per cent was eliminated unchanged in the urine. According to the result of Jaffé's experiment, muconic acid or any of its derivatives might be regarded as intermediary products during the process of the vital cleavage of tyrosine and phenylalanine. Fromherz and Hermanns believe that these last two acids decompose through a dual path as follows:



Previously in the laboratory of Jaffé, Kotake carried out a feeding experiment with tyrosine. The urine of a rabbit was examined for muconic acid or any of its derivatives, with negative result. Later Kotake, working on investigations of the fate of bivalent fatty acids in the animal body, found that adipic acid was with difficulty subjected to animal combustion and that a greater part of the acid administered was eliminated unchanged in the urine.

In chemical constitution muconic acid differs from adipic acid only in that the former possesses two double linkages, while the latter is a saturated compound having the following formulas.

$$COOH - CH = CH - CH = CH - COOH$$
(muconic acid)

$$COOH - CH_2 - CH_2 - CH_2 - CH_2 - COOH$$
(adipic acid)

It is well known that there is considerable difference between saturated and unsaturated compounds in their behavior towards many chemical reagents. But so far this difference has not been verified in animal oxidation in which the combustibility of substances is chiefly decided by other physiological relations. Consequently the easy combustibility of muconic acid in the animal body contrary to the behavior of adipic acid, appears to indicate an important physiological meaning. It is therefore desirable that the investigations as to the fate of both acids—muconic and

adipic—in the animal body should be simultaneously and exactly repeated, so that both results may be accurately compared. Following this suggestion of Kotake's, I undertook to repeat the experiments of Jaffé and of Kotake.

EXPERIMENTAL.

Muconic and adipic acids used in the experiments were synthetically prepared in the following ways.

Mucic acid obtained by the action upon milk sugar was, at first, converted into dichloromuconic acid by means of phosphorus pentachloride, according to the description of Bode, and then into hydromuconic acid. Finally, the latter acid was changed into muconic acid over dibromomuconic acid according to Limpricht and Marquardt.

Adipic acid was prepared according to Markownikoff by the action of nitric acid upon cyclohexane which was obtained through the reduction of benzene according to Sabatier and Senderens.

The experiments were carried out on rabbits fed with okara (a residue obtained from bean curds). The animals were subcutaneously injected with muconic and adipic acids, previously neutralized by means of sodium carbonate. Each specimen of the urine collected during the following 24 hours was examined.

It may be probable that adipic acid is eventually oxidized into oxalic acid, this oxidation taking place according to the β-oxidation of Knoop.

$$\begin{array}{c} COOH-CH_2-CH_2-CH_2-CH_2-COOH \\ \downarrow \\ (COOH-CH_2-CH_2-COOH) \\ \downarrow \\ COOH-COOH \end{array}$$

Recently Hensel and Riesser found that the surviving liver forms acetone bodies from muconic acid.

Consequently in my experiments with these acids I intended, on the one hand, to estimate the rate of their vital cleavage and on the other, to investigate the increase of oxalic acid in the urine, and further to examine the formation of acetone bodies from adipic acid in the surviving liver. The quantitative determination was carried out by the process of Autenrieth and Barth, the acid being isolated in the form of calcium salt and then converted into calcium oxide.

The mother liquor, which reacted alkaline on account of the presence of ammonia, was evaporated on the water bath to about 50 cc., and after cooling strongly acidified by means of hydrochloric acid, and was extracted fifteen times with ether. The ether was distilled to about 50 cc. and then decanted into a beaker and allowed to stand in the air. Muconic or adipic acid, eliminated unchanged in the urine, crystallized out. This was carefully purified and weighed.

All results obtained are summed up in Tables I to VIII.

TABLE I.

Rabbit A, Weight about 3,450 Gm.

Date.	Muconic acid given.	Muconic acid in urine.	Oxalic acid in urine.	Urine.	Specific gravity.	Reaction.	Remarks.
	gm.	*gm.*	*gm.*	*cc.*			
Nov. 3			0.0084	218	1.013	Slightly alkaline.	
" 4			0.0088	195	1.015	" "	
" 5	0.8						Muconic acid injected subcutaneously in the form of the sodium salt.
" 6			0.0085	218	1.015	Slightly acid.	
" 24	0.8						Same as for Nov. 5.
" 25			0.0087	194	1.019	" "	
Mar. 9	0.8						Same as for Nov. 5.
" 10		0.5904	0.0040	153	1.024	Neutral.	

All specimens of muconic acid obtained from the urine crystallized out in prisms of a brownish color. They were dissolved together in water with sodium carbonate, decolorized with animal charcoal, and then thoroughly acidified with sulfuric acid whereupon muconic acid separated out. It was filtered off, recrystallized from alcohol (80 per cent), and identified by analysis.

0.2351 gm. substance (dried at 100°C. in vacuum) gave 0.4356 gm. CO_2 and 0.0935 gm. H_2O.

	Calculated for $C_6 H_6 O_4$: *per cent*	Found: *per cent*
C	50.70	50.53
H	4.22	4.45

TABLE II.

Rabbit B, Weight about 2,750 Gm.

Date.	Muconic acid given.	Muconic acid in urine.	Oxalic acid in urine.	Urine.	Specific gravity.	Reaction.	Remarks.
	gm.	*gm.*	*gm.*	*cc.*			
Apr. 30	0.8						Muconic acid injected subcutaneously at once in the form of the sodium salt.
May 1		0.5840	0.0069	210	1.020	Slightly acid.	
" 2			0.0067	265	1.014	Slightly alkaline.	
" 7–8			0.0143	345	1.016	" "	Oxalic acid estimated in urine collected during 36 hrs.
" 9	0.8						Muconic acid dissolved in 20 cc. of water, neutralized by sodium carbonate, divided into four portions, and each portion injected subcutaneously at intervals of 3 hrs.
" 9–10		0.5928	0.0146	145	1.025	Slightly acid.	Urine of the 24 hrs. after the first injection and urine of the following 12 hrs. separately collected; muconic acid was estimated in each specimen. Oxalic acid estimated at one time in all the urine of the 36 hrs.
		Trace.		132	1.015	Neutral.	
" 10–11			0.0148	240	1.015	Slightly alkaline.	
				115	1.020	" "	

All specimens of adipic acid obtained from the urine were combined, recrystallized, and identified by analysis.

0.2058 gm. substance (dried at 105–110°C.) gave 0.3712 gm. CO_2 and 0.1305 gm. H_2O.

	Calculated for $C_6H_{10}O_4$: *per cent*	Found: *per cent*
C	49.29	49.19
H	6.90	7.10

TABLE III.

Rabbit C, Weight about 3,400 Gm.

Date.	Muconic acid given.	Muconic acid in urine.	Urine.	Specific gravity.	Reaction.	Remarks.
	gm.	*gm.*	*cc.*			
May 11	0.8					
" 12		0.5716	114	1.022	Slightly alkaline.	
" 13, 12 hrs. later.		Trace.	184	1.015	" "	
" 14, 24 hrs. later.		Not present.	125	1.016	" "	
" 15	0.8	0.340			" "	Muconic acid in form of its sodium salt was dissolved in 20 cc. of water and given by stomach tube.
" 16		Not present.	230	1.015	" "	
" 17			157	1.015	" "	

In every experiment with muconic or adipic acid I have estimated the oxalic acid in the feces of the 24 hours before and after the injection of one acid or the other, but found no difference in any two specimens (data omitted).

As the tables show, my conclusions, contrary to those of Jaffé, indicate that muconic acid was for the greater part eliminated unchanged in the urine. The result obtained with adipic acid was similar to that of Kotake, and the greater part of the adminis-

TABLE IV.

Rabbit D, Weight about 2,700 Gm.

Date.	Adipic acid given.	Adipic acid in urine.	Oxalic acid in urine.	Urine.	Specific gravity.	Reaction.	Remarks.
	gm.	*gm.*	*gm.*	*cc.*			
June 10			0.0048	180	1.012	Slightly alkaline.	
" 11	2.0						Adipic acid injected subcutaneously at once in the form of its sodium salt.
" 12		1.1035	0.0130	146	1.025	" "	
" 13			0.0060	198	1.012	" "	
" 14			0.0042	180	1.011	" "	
" 21	2.0						Same as for June 11.
" 22		0.9746	0.0190	125	1.025	Slightly acid.	
" 23			0.0042	215	1.013	Slightly alkaline.	
" 24			0.0064	192	1.013	" "	
" 29	2.0						Same as for June 11.
" 30		1.0968	0.0189	127	1.025	Slightly acid.	
July 1			0.0042	213	1.013	Slightly alkaline.	
" 2			0.0051	167	1.017	" "	
" 6	0.8						Adipic acid was dissolved in 20 cc., neutralized by sodium carbonate, divided into four portions, each portion injected simultaneously at intervals of 3 hrs.
" 7		0.4368		325	1.015	Slightly acid.	The urine was collected during 36 hrs. after the first injection, and adipic acid in it was estimated.
" 10	0.8						Adipic acid injected subcutaneously at once in the form of its sodium salt.
" 11		0.4789	0.0084	135	1.020	Slightly alkaline.	
" 12			0.0077	137	1.018	" "	
" 13			0.0046	133	1.012	" "	

TABLE V.

Rabbit E, Weight about 3,450 Gm.

Date.	Adipic acid given.	Adipic acid in urine.	Oxalic acid in urine.	Urine.	Specific gravity.	Reaction.	Remarks.
	gm.	*gm.*	*gm.*	*cc.*			
June 11			0.0040	198	1.012	Slightly alkaline.	
" 12	2.0						Adipic acid injected subcutaneously at once in the form of its sodium salt.
" 13		1.1868	0.0144	122	1.028	" "	
" 14			0.0047	175	1.014	" "	
" 15			0.0045	175	1.014	" "	
" 20	0.8						Adipic acid injected subcutaneously at four times as before.
" 21		0.5104	0.0072	247	1.021	" "	
" 22			0.0053	180	1.012	" "	
" 23			0.0051	168	1.013	" "	

TABLE VI.

Rabbit A, Weight about 3,450 Gm.

Date.	Adipic acid given.	Adipic acid in urine.	Oxalic acid in urine.	Urine.	Specific gravity.	Reaction.	Remarks.
	gm.	*gm.*	*gm.*	*cc.*			
Feb. 11			0.0048	167	1.020	Slightly acid.	
" 12			0.0058	182	1.016	Neutral.	
" 13	0.8						Adipic acid injected subcutaneously at once in the form of its sodium salt.
" 14		0.5826	0.0056	182	1.018	Slightly acid.	
" 15			0.0048	190	1.015	Neutral.	
			0.0042	184	1.014	Slightly acid.	

TABLE VII.

Rabbit F, Weight about 2,830 Gm.

Date.	Adipic acid given.	Adipic acid in urine	Oxalic acid in urine.	Urine.	Specific gravity.	Reaction.	Remarks.
	gm.	*gm.*	*gm.*	*cc.*			
Mar. 24			0.0053	182	1.018	Slightly acid.	
" 25	0.8						Adipic acid injected subcutaneously at once in the form of its sodium salt.
" 26		0.5624	0.0064	165	1.025	Neutral.	
" 27			0.0058	170	1.018	Slightly acid.	
" 28			0.0055	152	1.020	" "	

TABLE VIII.

Rabbit B, Weight about 2,750 Gm.

Date.	Adipic acid given.	Adipic acid in urine.	Oxalic acid in urine.	Urine.	Specific gravity.	Reaction.	Remarks.
	gm.	*gm.*	*gm.*	*cc.*			
May 15			0.0072	270	1.013	Slightly alkaline.	
" 16	0.8						Adipic acid injected subcutaneously at once in the form of its sodium salt.
" 17		0.5890	0.0077	253	1.015	" "	
" 18			0.0055	255	1.014	" "	
" 19			0.0067	255	1.014	" "	

tered acid appeared again in the urine. For the sake of clearness, these results are collected in Tables IX and X.

Finding that muconic acid administered to a rabbit was almost completely oxidized in the body so that only 1 per cent of it appeared unchanged in the urine, Jaffé assumed that, if benzene is injected into a rabbit, 25 to 30 per cent of it may be decomposed

over muconic acid. But my results to the contrary make Jaffé's assumption untenable. We are obliged to assume that the benzene is converted to but a slight extent if at all to muconic acid in the animal body.

TABLE IX.

Muconic Acid.

Date.	Rabbit.	Acid given.	Acid in urine.		Remarks.
		gm.	*gm.*	*per cent*	
Mar. 9–10	A	0.8	0.5904	73.08	Injected at one time.
Apr. 30–May 1	B	0.8	0.5840	73.00	" " " "
May 9–10	"	0.8	0.5928	74.10	" " four times.
" 11–12	C	0.8	0.5716	71.45	" " " "
" 15–16	"			72.91	Average.
		0.8	0.3490	43.63	Introduced in stomach.

TABLE X.

Adipic Acid.

Date.	Rabbit.	Acid given.	Acid in urine.		Remarks.
		gm.	*gm.*	*per cent*	
June 11–12	D	2.0	1.1035	55.18	Injected at one time.
" 21–22	"	2.0	0.9746	48.73	" " " "
" 29–30	"	2.0	1.0968	54.84	" " " "
July 6– 7	"	0.8	0.4368	54.60	" " four times.
" 9–10	"	0.8	0.4798	59.86	" " one time.
" 12–13	E	2.0	1.1868	9.34	" " " "
" 20–21	"	0.8	0.5104	63.80	" " four times.
Feb. 13–14	A	0.8	0.5826	72.83	" " one time.
Mar. 25–26	F	0.8	0.5624	70.30	" " " "
May 16–17	B	0.8	0.5890	73.63	" " " "
Average.....				61.31	

The physiological relations of muconic acid or any of its derivatives, especially their relation to aromatic amino-acids might be decided through further investigations. In this paper I wish only to call attention to the fact that the decomposition of muconic acid itself in the animal organism occurs no more readily than that

of adipic acid, which so far is not regarded as an intermediary product of physiological metabolism.

The administration of adipic acid is always followed by the increase of oxalic acid in the urine, whereas this increase is not detected in the experiment with muconic acid.

The investigations concerning the decomposition of adipic acid in the surviving liver are not yet completed. The results will be published later.

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EXPERIMENTS ON THE UTILIZATION OF NITROGEN, CALCIUM, AND MAGNESIUM IN DIETS CONTAINING CARROTS AND SPINACH.*

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Albu and Neuberg remark that, "The calcium from plant foods, in which it is more abundant, is less readily absorbed than that from meat nourishment." Such statements have often been based upon the amounts of calcium excreted by the kidney under different dietary conditions. That this is an unsafe criterion for the extent of absorption has been shown by Voit, who proved that calcium may be absorbed from the small intestine and then secreted into the large intestine. Likewise, Forster showed that as much as 60 per cent of the calcium in the food may be absorbed and only a very small portion of this amount excreted by the kidneys. Kochmann and his pupil Petzsch carried out extensive studies upon the effect of diets of protein, fat, and carbohydrate upon the excretion of calcium and magnesium in dogs. They concluded that the amount of calcium necessary for maintaining a positive calcium balance depends upon the character of the diet and should be specifically determined for different types of food intake.

Courtney, Fales, and Bartlett cite the fact that the feeding of green vegetables to young infants is becoming a common practice; and refer to the generally accepted view that the value of such dietary components is attributable to the effect on the mineral metabolism.

Many factors are thus seen to be involved in the problems here at issue. The present investigations aim to evaluate somewhat more critically the behavior of calcium and magnesium as dietary components. To this end the balance of these elements in the body has been determined in a series of nutrition experiments on animals.

* The data in this paper are taken from the dissertation presented by Harry B. McClugage for the Degree of Doctor of Philosophy, Yale University, 1918

Methods.

The general plan consisted of metabolism experiments in which the intake and output of nitrogen, calcium, and magnesium were ascertained and the corresponding balances calculated, under diverse dietary conditions.

Previous investigations in this laboratory had shown that owing to the small amount of calcium eliminated in dog's urine, it was necessary to use for analysis a larger volume of urine than called for in the McCrudden method of determination. This was accomplished by concentrating the acidified urine on the water bath to a small volume. The filtered fluid was treated as directed by McCrudden, allowed to stand over night, then filtered. The precipitate was washed several times with 0.5 per cent ammonium oxalate solution, ignited with the filter paper, dissolved in dilute hydrochloric acid solution, filtered, and reprecipitated according to McCrudden's method for the estimation of calcium in human urine. The final precipitates were ignited and weighed as calcium oxide.

The combined filtrates were taken for the magnesium estimation and treated as is called for by McCrudden's directions to be used when iron may be present. The calcium and magnesium in the ash of all the foods and the feces were determined by the original McCrudden technique. Nitrogen was estimated by a Kjeldahl method.

The meat fed was lean beef purchased in large quantities, cleaned as free as possible from all fat and connective tissue, hashed, put up in weighed amounts in waxed paper, and kept frozen in a refrigerator.

The dried skimmed milk fed was Merrell-Soule Company's "Klim."

The vegetables fed were bought in large quantities and the thoroughly washed edible portions were dried on steam radiators at a temperature of 65–70°C. for several days. This desiccation, in the case of the carrots, was facilitated by cutting them into thin slices. After drying until it could be easily powdered, each vegetable was ground finely in a mill several times in order to obtain well mixed samples. Table I shows the composition of the components of the diets.

In the cases when the cooked vegetable was fed, the dry, powdered, daily portions were added to boiling water and boiled for half an hour, the water used being added to the diet, so as to prevent any loss of extracted salts.

The animals were not catheterized. Inasmuch as the periods were fairly long ones whatever urine was retained in the bladder would cause only slight errors in the final results. The periods were marked off in the case of the feces by mixing approximately 1 gm. of carmine with the food.

TABLE I.

Composition of the Components of the Diets.

	N	Fat.	Carbohydrate.	Ca	Mg
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
Meat I.	3.36	2.6	—	0.011	0.023
Cracker meal	1.66	6.0	73	0.027	0.019
Dried skimmed milk (Klim)	5.66	—	56.8	1.23	0.232
Agar	—	—	—	0.740	0.210
Dried Spinach 1*	4.26	—	—	0.595	0.642
" " 2*	5.13	—	—	0.995	0.854
" Carrots 1†	1.15	—	—	0.260	0.071
" " 2†	1.84	—	—	0.298	0.127

* Approximately 15 per cent of moist weight.
† " 12 " " " " "

EXPERIMENTAL.

The relative utilization of nitrogen, calcium, and magnesium in diets containing spinach and carrots was studied in comparison with rations of meat plus dried milk and meat plus calcium carbonate. Tables II and III show the composition of the diets.

Two grown female dogs were used. Dog A weighed 15.6 kilos at the beginning of the experiment and 15.0 at the end. Dog B at the beginning of the experiment weighed 9.6 kilos and 10.2 at the end. The dogs were allowed distilled water freely throughout the experiments. Agar-agar was fed in all diets, except during the periods when carrots were given, to provide bulk to the feces and to insure daily evacuation of the bowels, thus lessening the reabsorption. In the case of the carrots, the addition of agar-agar to the diet seemed likely to provide too large a bulk to the food;

TABLE II.

Composition of Daily Diets. Dog A.

		N	Fat.	Carbohydrate.	Ca	Mg	Calories (approximate).
Diet A_1.							
	gm.	*gm.*	*gm.*	*gm.*	*mg.*	*mg.*	
Meat I	100	3.36	2.6	—	11	23	108
Cracker meal	100	1.66	6.0	73.0	27	19	390
Lard	30	—	30.0	—	—	—	279
Sugar	20	—	—	20.0	—	—	80
Agar	10	—	—	—	74	21	—
$CaCO_3$	0.535	—	—	—	214	—	—
Total		5.02	38.6	93.0	326	63	857
Diet A_2.							
Meat I	100	3.36	2.6	—	11	23	108
Cracker meal	40	0.66	2.4	29.2	11	8	156
Lard	40	—	40.0	—	—	—	372
Sugar	30	—	—	30.0	—	—	120
Agar	10	—	—	—	74	21	—
Dried milk	17.6	1.00	—	10.0	214	23	67
Total		5.02	45.0	69.2	310	75	823
Diet A_3.							
Meat I	100	3.36	2.6	—	11	23	108
Lard	40	—	40.0	—	—	—	372
Carrots 1	82.7	0.95	—		214	58	300
Total		4.31	42.6		225	81	780
Diet A_4.							
Meat I	100	3.36	2.6	—	11	23	108
Cracker meal	20	0.33	1.2	14.6	5	4	78
Lard	50	—	50.0	—	—	—	465
Sugar	40	—	—	40.0	—	—	160
Agar	10	—	—	—	74	21	—
Spinach 2	21.5	1.10	—	—	214	183	28
Total		4.79	53.8	54.6	304	231	839
Diet A_5.							
Meat I	140	4.70	3.7	—	15	31	151
Lard	50	—	50.0	—	—	—	465
Sugar	60	—	—	60.0	—	—	240
Agar	10	—	—	—	74	21	—
Total		4.70	53.7	60.0	89	52	856

TABLE III

Composition of Daily Diets. Dog B.

		N	Fat.	Carbohydrate.	Ca	Mg	Calories (approximate).
Diet B_1.							
	gm.	*gm.*	*gm.*	*gm.*	*mg.*	*mg.*	
Meat I	100	3.36	2.6	—	11	23	108
Lard	30	—	30.0	—	—	—	279
Sugar	25	—	—	25.0	—	—	100
Agar	10	—	—	—	74	21	—
Dried milk	17.6	1.00	—	10.0	214	23	67
Total		4.36	32.6	35.0	299	67	554
Diet B_2.							
Meat I	100	3.36	2.6	—	11	23	108
Lard	30	—	30.0	—	—	—	279
Sugar	30	—	—	30.0	—	—	120
Agar	10	—	—	—	74	21	—
Spinach 1	36.0	1.53	—	—	214	231	26
Total		4.89	32.6	30.0	299	275	533
Diet B_3.							
Meat I	100	3.36	2.6	—	11	23	108
Cracker meal	60	1.00	3.6	44.0	16	12	234
Lard	20	—	20.0	—	—	—	186
Sugar	20	—	—	20.0	—	—	80
Agar	10	—	—	—	74	21	—
$CaCO_3$	0.535	—	—	—	214	—	—
Total		4.36	26.2	64.0	315	56	608
Diet B_4.							
Meat I	100	3.36	2.6	—	11	23	108
Lard	20	—	20.0	—	—	—	186
Carrots 1	82.7	0.95	—	—	214	58	300
Total		4.31	22.6		225	81	594
Diet B_5.							
Meat I	100	3.36	2.6	—	11	23	108
Lard	20	—	20.0	—	—	—	186
Carrots 2	71.85	1.32	—	—	214	91	300
Total		4.68	22.6		225	114	594

TABLE III—*Concluded.*

		N	Fat.	Carbohydrate.	Ca	Mg	Calories (approximate).
Diet B_6.							
	gm.	*gm.*	*gm.*	*gm.*	*mg.*	*mg.*	
Meat I	100	3.36	2.6	—	11	23	108
Lard	30	—	30.0	—	—	—	279
Sugar	30	—	—	30.0	—	—	120
Agar	10	—	—	—	74	21	—
Spinach 2	21.5	1.10	—	—	214	183	28
Total		4.46	32.6	30.0	299	227	535
Diet B_7.							
Meat I	130	4.37	3.4	—	14	30	140
Lard	30	—	30.0	—	—	—	279
Sugar	35	—	—	35.0	—	—	140
Agar	10	—	—	—	74	21	—
Total		4.37	33.4	35.0	88	51	559

hence it was omitted during these periods. In no case were there intestinal disturbances of any sort. Throughout the experiments the urines showed an acid reaction to litmus. The length of all the experimental periods was 7 days. The average daily balances are shown in Tables IV and VI; the percentage utilizations, in Tables V and VII.

Utilization of Nitrogen.

In the case of Dog A there was a negative nitrogen balance throughout the latter half of the experiment, Periods 6 to 10, probably due, in major part, to the poor utilization of the nitrogen of the vegetable portion of the diets. A study of the percentage utilization of nitrogen, Table V, shows plainly that in the case of the vegetables it was not so favorable. The average utilization upon meat plus cracker meal and meat plus milk was 89 per cent while the average with diets of meat plus vegetables was 78 per cent. This difference in utilization is the more striking when it is noted that only about one-fourth of the nitrogen came from the vegetable, the remaining three-fourths from the well utilized meat.

TABLE IV.

Average Daily Outputs and Balances. Dog A.

Period.	Weight.	Nitrogen.					Calcium.					Magnesium.					Diet.
		Output.			Intake, food.	Balance.	Output.			Intake, food.	Balance.	Output.			Intake, food.	Balance.	
		Urine.	Feces.	Total.			Urine.	Feces.	Total.			Urine.	Feces.	Total.			
	kg.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	
1	15.6	4.37	0.55	4.92	5.02	+0.10	16	460	476	326	−150	27	57	84	63	−21	A_1 $CaCO_3$.
2	15.6	4.18	0.63	4.81	5.02	+0.21	18	422	440	326	−114	33	57	90	63	−27	A_1 $CaCO_3$.
3	15.4	4.44	0.53	4.97	5.02	+0.05	13	412	425	310	−115	20	65	85	75	−10	A_2 milk.
4	15.6	4.38	0.54	4.92	5.02	+0.10	10	361	371	310	− 61	22	51	73	75	+ 2	A_2 "
5	15.6	4.49	0.51	5.00	5.02	+0.02	11	447	458	310	−148	23	64	87	75	−12	A_2 "
6	15.2	3.99	1.14	5.13	4.31	−0.82	9	437	446	225	−221	25	117	142	81	−61	A_3 raw carrots.
7	15.2	3.85	1.08	4.93	4.31	−0.62	8	427	435	225	−210	23	111	134	81	−53	A_3 cooked carrots.
8	15.0	4.08	1.00	5.08	4.79	−0.29	5	567	572	304	−268	28	194	222	231	+ 9	A_4 raw spinach.
9	15.0	4.23	0.79	5.02	4.79	−0.23	5	572	577	304	−273	37	183	220	231	+11	A_4 cooked spinach.
10	15.0	4.24	0.48	4.72	4.70	−0.02	12	267	279	89	−190	27	44	71	52	−19	A_5 meat.

TABLE V.

Nitrogen, Calcium, and Magnesium Metabolism. Percentage Utilization. Dog A (7 Day Period).

Period.	Diet.	Nitrogen.			Calcium.			Magnesium.		
		Intake, food.	Output, feces.	Utilization.	Intake, food.	Gain (+) or loss (−) of total intake.		Intake, food.	Gain (+) or loss (−) of total intake.	
		gm.	*gm.*	*per cent*	*gm.*	*gm.*	*per cent*	*gm.*	*gm.*	*per cent*
4	A_2 milk	35.1	3.76	89	2.170	−0.438	−20	0.525	+0.012	+ 2
2	A_1 $CaCO_3$	35.1	4.40	87	2.282	−0.805	−35	0.441	−0.184	−42
3	A_2 milk	35.1	3.69	90	2.170	−0.811	−37	0.525	−0.066	−12
5	A_2 "	35.1	3.54	90	2.170	−1.029	−47	0.525	−0.082	−16
1	A_1 $CaCO_3$	35.1	3.88	89	2.282	−1.051	−47	0.441	−0.156	−35
8	A_4 spinach	33.5	6.98	79	2.128	−1.880	−88	1.617	+0.061	+ 4
9	A_4 "	33.5	5.52	84	2.128	−1.903	−89	1.617	+0.080	+ 5
7	A_3 carrots	30.2	7.53	75	1.575	−1.472	−94	0.567	−0.376	−66
6	A_3 "	30.2	8.00	74	1.575	−1.541	−98	0.567	−0.426	−75
10	A_5 meat	32.9	3.38	90	0.623	−1.315	—	0.364	−0.139	−38

Similar results were obtained with Dog B. The animal was in positive nitrogen balance throughout the experiment, because the proportion of nitrogen to body weight was larger in this case. But here, likewise, there was poor utilization of the nitrogen components of the vegetables. The average utilization for meat plus cracker meal and meat plus milk was 94 per cent, while the average for meat plus vegetables was 85 per cent (Table VII).

That the utilization of nitrogen was not bettered by the cooking of the vegetables is shown by a comparison of Period 6 with Period 7 (Table V) and Period 4 with Period 9 (Table VII).

These facts are concordant with the current knowledge concerning the utilization of proteins from certain vegetable sources. Mendel and Fine, who have given a résumé of the literature, found that the proteins of legumes are relatively poorly utilized.

The calculations have been based upon the relation of fecal nitrogen to ingested nitrogen. While this relation is not an absolutely safe criterion of utilization, nevertheless, it may suffice for the approximate comparison here desired.

TABLE VI.

Average Daily Outputs and Balances. Dog B.

Period.	Weight.	Nitrogen.					Calcium.					Magnesium.					Diet.
		Output.			Intake, food.	Balance.	Output.			Intake, food.	Balance.	Output.			Intake, food.	Balance.	
		Urine.	Feces.	Total.			Urine.	Feces.	Total.			Urine.	Feces.	Total.			
	kg.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	
1	9.7	3.31	0.27	3.58	4.36	+0.78	10	400	410	299	−111	30	50	80	67	−13	B_1 milk.
2	9.6	3.32	0.25	3.57	4.36	+0.79	6	370	376	299	− 77	21	51	72	67	− 5	B_1 "
3	9.7	3.82	0.77	4.59	4.89	+0.30	4	547	551	299	−252	25	231	256	275	+19	B_2 raw spinach.
4	9.7	3.81	0.69	4.50	4.89	+0.39	5	433	438	299	−139	25	212	237	275	+38	B_2 " "
5	9.8	3.65	0.27	3.92	4.36	+0.44	8	376	384	315	− 69	20	44	64	56	− 8	B_3 $CaCO_3$.
6	10.0	3.20	0.28	3.48	4.36	+0.88	11	420	431	315	−116	20	44	64	56	− 8	B_3 $CaCO_3$.
7	10.2	3.33	0.64	3.97	4.31	+0.34	10	375	385	225	−160	20	109	129	81	−48	B_4 raw carrots.
8	10.0	3.76	0.75	4.51	4.68	+0.17	8	354	362	225	−137	26	86	112	114	+ 2	B_5 cooked carrots.
9	10.0	3.71	0.62	4.33	4.46	+0.13	5	545	550	299	−251	24	158	182	227	+45	B_6 " spinach.
10	10.0	3.50	0.24	3.74	4.36	+0.62	7	321	328	299	− 29	26	45	71	67	− 4	B_1 milk.
11	10.0	3.48	0.26	3.74	4.37	+0.63	6	184	190	88	−102	19	41	60	51	− 9	B_7 meat.

TABLE VII.

Nitrogen, Calcium, and Magnesium Metabolism. Percentage Utilization. Dog B (7 Day Period).

Period.	Diet.	Nitrogen.			Calcium.			Magnesium.		
		Intake, food.	Output, feces.	Utilization	Intake, food	Gain (+) or loss (−) of total intake.		Intake, food.	Gain (+) or loss (−) of total intake.	
		gm.	*gm.*	*per cent*	*gm.*	*gm.*	*per cent*	*gm.*	*gm.*	*per cent*
10	B_1 milk	30.5	1.75	95	2.093	−0.205	−10	0.469	−0.026	− 6
5	B_3 $CaCO_3$	30.5	1.86	94	2.205	−0.483	−22	0.392	−0.059	−15
2	B_1 milk	30.5	1.77	94	2.093	−0.542	−26	0.469	−0.030	− 6
1	B_1 "	30.5	1.88	94	2.093	−0.777	−37	0.469	−0.088	−19
6	B_3 $CaCO_3$	30.5	1.98	93	2.205	−0.813	−37	0.392	−0.061	−16
4	B_2 spinach	34.2	4.86	86	2.093	−0.971	−46	1.925	+0.259	+13
8	B_5 carrots	32.8	5.17	83	1.575	−0.963	−61	0.798	+0.016	+ 2
7	B_4 "	30.2	4.45	87	1.575	−1.115	−73	0.567	−0.339	−60
9	B_6 spinach	31.2	4.32	87	2.093	−1.753	−84	1.589	+0.318	+20
3	B_2 "	34.2	5.42	84	2.093	−1.766	−85	1.925	+0.132	+ 7
11	B_7 meat	30.6	1.75	95	0.616	−0.713	—	0.357	−0.061	−17

Utilization of Calcium.

Both Dog A and Dog B were in negative calcium balance throughout the experiments. Little that is definite can be said in regard to the calcium excreted by the kidneys. While a study of Tables IV and VI shows a lowered excretion of calcium in the urine during those periods when spinach and carrots furnished the greater portion of the ingested calcium, this decrease is very insignificant in relation to the total calcium excreted.

The balance of calcium reveals more interesting facts. In the case of Dog A, the average *percentage loss* of the intake shows the following results: with milk in the diet, 34 per cent; with calcium carbonate, 41 per cent; with spinach, 88 per cent; with carrots, 96 per cent. With Dog B the percentage losses of the intake were smaller throughout; but here again there were large differences in the utilization of the calcium on the various diets. The average percentage of the calcium intake lost was: for milk, 18 per cent; calcium carbonate, 29 per cent; carrots, 67 per cent; spinach, 72 per cent. These results show plainly the difference in the degree of utilization of the calcium of the diets. The calcium in calcium

carbonate and in milk was assimilated to about the same degree though somewhat better in the latter case. This is in accordance with previous investigations; for instance, Givens and Mendel found that milk is more effective for calcium retention than is calcium lactate. In regard to the calcium from the vegetable sources evidently the utilization was very poor. This low assimilation of calcium ran parallel with the poor utilization of nitrogen. Here again the cooking of the vegetables showed no beneficial effect.

There are several factors involved in the poor utilization of calcium. In the first place, there is the question of deficient absorption from the intestine. Bertram states that the absorption of calcium in the case of vegetable foods is poorer than in the case of animal foods. The low calcium retention is, however, not necessarily an outcome of poor absorption, for the possibility of secretion into the large intestine after absorption must be taken into consideration. The bulk of indigestible residue in the diet, causing a fairly rapid evacuation of the bowel, may tend to decrease the amount of reabsorption. It is to be noted that during the periods when the vegetables were fed, the mass of the feces was greatly increased—to the extent of 90 to 100 per cent, on an average. The increased calcium loss was not proportionately large but was greater during these periods of maximal fecal output.

Utilization of Magnesium.

The magnesium balance of Dog A was negative except for a small positive balance in Periods 4, 8, and 9 (Table IV). However, during Periods 8 and 9, when spinach was fed there was a much larger intake of magnesium, which probably accounts for this small positive balance. In the case of Dog B there was a positive magnesium balance during Periods 3, 4, 8, and 9 (Table VI) during which the intakes of magnesium were relatively large. However, by a comparison of the relative percentage losses of the intakes (Table V, Periods 3 and 5 with Periods 6 and 7; Table VII, Periods 1 and 2 with Period 7) it can be noticed that when the intake is of the same order of magnitude, the magnesium from the vegetable diets is comparatively poorly utilized.

Interrelation of Calcium and Magnesium.

The relation of calcium to magnesium in the urine of Dog A varied throughout, ranging from 1 : 1.5 to 1 : 7. This change in the ratio is due to the variation in the urinary calcium since the amount of magnesium excreted by the kidneys remained fairly constant throughout. This was likewise true in the urine of Dog B where the ratio ranged from 1 : 2 to 1 : 5, the magnesium remaining constant, with varying amounts of calcium.

In regard to the feces, it appears that a large excretion of calcium is accompanied by a large output of magnesium, and contrariwise. But the varying magnitudes of the ingested magnesium do not permit definite conclusions.

DISCUSSION.

Constant body weight, the nitrogen balance, general appearance, and activity are not in themselves an index of true nutritive condition. Thus, it is recognized that apparently an animal may be in good bodily state while there is, nevertheless, a steady loss of some essential substance necessary for the prolonged maintenance of health. This fact is illustrated by the present experiments in which the animals showed only slight changes in the body weight, were in positive nitrogen balance, apparently in a state of well-being, while there was a continual loss of calcium from the body, as shown by the balance of intake and output.

The special need for calcium and possibly other inorganic salts is at length beginning to be recognized and taken into account in dietary plans. This necessity is an especially weighty factor to be considered during the period of growth. The importance of calcium as a structural element for the growing bones and teeth is one to be emphasized. Calcium has been supplied by the use of milk in the diet. Certain present day factors, for example the possible shortage in the milk supply and the problem of expense, raise the question as to other physiologically available sources of calcium.

Analyses have shown that green vegetables are comparatively rich in this element and, accordingly, additions of these substances to the diet have been proposed. Thus, Courtney, Fales, and Bartlett state: "The addition of green vegetables to the

dietary of young infants is becoming a common practice. It is generally believed that the value of this is due to the effect on the mineral metabolism." They report that in children showing a delayed development improvement has been brought about by such additions, and they conclude, on the basis of the mineral content of a number of vegetables, that spinach is the best one to provide a salt addition.

These authors recognize the fact that balance experiments are necessary in order to study this problem. They have been undertaken in the present studies. The results obtained tend to show that the calcium of the vegetables, spinach and carrots, is not so well utilized as is the calcium in milk or in calcium carbonate. Therefore, it would seem in so far as the *calcium* is concerned that, if milk were not available, the addition of a suitable calcium salt would be quite as rational a procedure as a vegetable addition. Furthermore, in order to add to the diet an appreciable amount of calcium from a vegetable source it would be necessary to feed large quantities of the vegetables, which in itself is a rather difficult procedure in the case of infants. This naturally does not speak against the vegetables as sources of vitamines, iron, or other desired dietary factors.

It appears in the light of the present experiments that the addition of such vegetables as were tested, to the diet, does not always yield a pronounced advantage to the calcium metabolism. It would, therefore, presumably be an unsafe procedure to use vegetables extensively as a dietary substitute for milk in the nutrition of children.

SUMMARY.

The calcium supply of the organism is normally derived from milk in greater abundance than from any other dietary sources. The comparative richness of green vegetables in calcium raises the question as to its availability for nutritive purposes. This problem cannot be solved by analyses of foods alone. Metabolism experiments involving the balance of intake and output are necessary. Such procedure has been followed in these studies. They show that when carrots and spinach were added to the diets of dogs the nitrogen was not well utilized; the calcium in milk was better utilized than that in calcium carbonate; the calcium

in spinach and carrots was poorly assimilated as compared with the calcium in milk. The conclusion seems justified that green vegetables should not be used extensively as a substitute for milk as the dietary source of calcium.

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THE RELATION OF THE QUALITY OF PROTEINS TO MILK PRODUCTION. IV.*

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In earlier publications[1] we have shown that there is a marked inequality in the efficiency of protein mixtures for milk production, making mammary secretion in this special sense comparable with growth. The growth of an animal as measured by live weight increments may be directly controlled by the quality of the proteins furnished, suspending, retarding, or accelerating cell expansion as the nature of the proteins ingested is altered. This condition can, of course, be secured only when all the other factors for adequate nutrition are provided. In mammary secretion this effect of a change in the quality of the proteins ingested is obscured, at least in studies of short duration, by the contributing factor of increased tissue catabolism, through which normal blood composition in respect to its nitrogen content can be maintained for considerable periods of time. It is this very fact, namely that there is an increased tissue catabolism in a milking animal during periods of inadequate protein ingestion—inadequate as to quality as well as to quantity—that makes it possible to measure the inequality of proteins for milk production.

While our work has been confined to dairy cows the recently reported work of Hoobler[2] on nursing mothers is in agreement with the results we have secured.

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[1] Hart, E. B., and Humphrey, G. C., *J. Biol. Chem.*, 1915, xxi, 239; 1916, xxvi, 457; 1917, xxxi, 445.

[2] Hoobler, B. R., *Am. J. Dis. Child.*, 1917, xiv, 105.

Up to the present we have confined our attention to studies planned to determine first, whether there was marked inefficiency for milk production in protein mixtures known to be inefficient for growth, and second, a study of some practical protein mixtures for milk production. The fact was established that the proteins of cereal grains, as maize and wheat, when supplemented with a roughage like corn stover, were strikingly less efficient for maintaining the nitrogen equilibrium of a milking animal than was a ration composed of milk proteins and corn stover. With the above rations fed on the same plane of protein intake it was further established that during short periods (4 weeks) of observation with dairy cows a negative nitrogen balance may prevail without reducing milk secretion appreciably. These are the fundamental facts relating to protein metabolism and milk secretion thus far established.

In addition to these fundamental facts, studies were made on the protein efficiency of certain common protein concentrates used to supplement a definite basal ration, but a changing roughage. With *corn stover* and corn-meal as a basal ration, gluten feed, the high protein concentrate from corn-meal, was measurably inferior as a protein supplement to either oil meal or distillers' grains. The two latter were practically equally efficient. With *clover hay* (medium red, *Trifolium pratense*) and corn-meal as the basal ration the inequality among these vegetable concentrates disappeared and gluten feed proteins were practically equal in supplementing power to those of oil meal, distillers' grains, or cottonseed meal.

This is a striking illustration of the fallacy of condemning a material because when used alone or in *certain* mixtures its proteins display a poor quality. This may not be true for *other mixtures*. The tremendous increase in the efficiency of the cereal proteins by supplementing them with either skimmed milk or whey is a striking example of this fact.[3] In these growth experiments with swine the efficiency of the cereal proteins has been raised from 25 to 65 per cent by the use of skimmed milk proteins and from 25 to 47 per cent by the use of whey proteins, which contributed but 15 per cent of the total nitrogen

[3] McCollum, E. V., *J. Biol. Chem.*, 1914, xix, 323. Hart, E. B., and Steenbock, H., *Wisconsin Exp. Station Bull. 291*, 1918.

intake. Although the nitrogen contributed by the whey was extremely small in amount, yet its positive supplementing effect on the cereal grain proteins was most marked. Osborne and Mendel[4] in their use of protein-free milk as a supplement to milk albumin rated the latter as a protein of very high efficiency, but this efficiency entirely disappeared when milk albumin was the sole source of nitrogen in the ration. This observation demonstrated the very effective supplementary capacity of the small amount of nitrogen contained in natural protein-free milk for such a protein as milk albumin.

During the past year we have continued the milk secretion studies, following the same plan as used heretofore, but this time using *alfalfa* (*Medicago sativa*) as the basal roughage, with corn-meal as the grain and the vegetable protein concentrates gluten feed, oil meal, distillers' grains (Ajax), and cottonseed meal. In these studies cottonseed meal was the least efficient, gluten feed and oil meal were of greater efficiency than cottonseed meal and of approximately equal effect, while distillers' grains were the most effective of these four protein concentrates. Here is another illustration of differences in the nitrogen efficiency of new combinations and how the nitrogen of alfalfa has influenced the utilization of these protein combinations.

EXPERIMENTAL.

The plan was to use a basal ration of alfalfa hay, corn silage, corn-meal, and starch to which would be added the protein concentrate. The basal ration was maintained constant in relation to its source and proportion of nutrients for any individual animal in the different periods, the only variables in succeeding periods being the concentrate and the amount of starch. Because of the abundance and constancy of energy, mineral materials, and vitamines in these rations we can with justice attribute the different effects observed to the proteins of the concentrate added. The concentrates and starch were supplied in such quantities as to make the plane of protein intake and net available energy uniform in the several periods.

[4] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1915, xx, 351; 1916, xxvi, 1; 1917, xxxi, 149.

The various rations were fed at a nutritive plane of approximately 1:8.4, or with a total protein intake of 10 per cent of the dry matter of the ration. Table I illustrates the proportion of the various feeds in the ration where an animal was receiving daily approximately 50 pounds of material, which contained 20.5 production therms and 2.32 pounds of digestible protein.

Two grade Guernsey cows and one pure bred Jersey were used for the work. They were not with calf. The animals were milked twice daily and exercised two or three times per week. Weights of the animals were taken weekly. The plan was to place each individual on any one of the rations for a period of 4 weeks with an immediate change to one of the other rations, thus involving each animal in 16 weeks of observation. A feeding period of 1 week preceded the quantitative collection of feces and urine.

TABLE I.

Source and Proportion of Nutrients Used.

Period.	Gluten feed.	Distillers' grains.	Oil meal.	Cottonseed meal.
	lbs.	*lbs.*	*lbs.*	*lbs.*
Alfalfa hay	7.00	7.00	7.00	7.00
Corn silage	28.00	28.00	28.00	28.00
Corn-meal	6.00	6.00	6.00	6.00
Concentrate	4.00	3.86	3.10	2.99
Starch	5.00	5.14	5.90	6.01

The urine and feces were analyzed daily for nitrogen, while a weekly analysis was made of a 7 day composite sample of milk.

Our earlier observations had shown that when a nutritive ratio of 1:8 was used, equivalent to a plane of total protein intake of approximately 9 to 10 per cent and the nutrients drawn from corn stover, corn-meal, and certain vegetable protein concentrates, a positive nitrogen balance could not be maintained. But with the roughage changed from corn stover to clover hay a positive nitrogen balance was maintained most of the time with practically the same nutritive ratio. Because of these facts it was planned to use a ration with a nutritive ratio of approximately 1:8.5 as it was essential for the animals to be in a negative nitrogen balance or just in nitrogen equilibrium in studies of this character. In the final adjustment of our ration the nutritive ratio

became fixed at 1:8.4. In this ration, this ratio (dry matter basis) was equivalent to approximately 8.6 per cent of digestible protein and 10 per cent of total protein. The composition of the rations used is shown in Table II.

TABLE II.

Composition of Ration.

	Weight.	Nitrogen.	Total N.	Production.	Digestible protein; nutritive ratio.
Gluten feed ration.					
	lbs.	*per cent*	*gm.*	*therms*	
Alfalfa hay	7	2.33	74.04	2.40	
Corn silage	28	0.28	35.39	4.64	
Corn-meal	6	1.44	39.22	5.38	2.32 lbs. digestible protein.
Gluten feed	4	4.74	86.07	3.17	
Starch	5	0.03	0.68	5.00	
Total	50		235.40	20.59	1:8.4
Oil meal ration.					
Alfalfa hay	7	2.33	74.04	2.40	
Corn silage	28	0.28	35.39	4.64	
Corn-meal	6	1.44	39.22	5.38	
Oil meal	3.10	6.09	85.71	2.44	
Starch	5.90	0.03	0.80	5.90	
Total	50		235.16	20.76	1:8.4
Distillers' grains (Ajax) ration.					
Alfalfa hay	7	2.33	74.04	2.40	
Corn silage	28	0.28	35.39	4.64	
Corn-meal	6	1.44	39.22	5.38	
Distillers' grains	3.86	4.84	84.82	3.05	
Starch	5.14	0.03	0.70	5.14	
Total	50		234.17	20.61	1:8.4
Cottonseed meal ration.					
Alfalfa hay	7	2.33	74.04	2.40	
Corn silage	28	0.28	35.39	4.64	
Corn-meal	6	1.44	39.22	5.38	
Cottonseed meal	2.99	6.26	85.13	2.25	
Starch	6.01	0.03	0.90	6.01	
Total	50		234.68	20.68	1:8.4

The four rations were much alike in content of production therms and total protein. Table II gives the composition of 50 pounds of the mixed ration. The animals were not fed 50 pounds of the ration daily, but that proportion which would meet the maintenance and energy requirements for their specific milk pro-

TABLE III.

Record of Nitrogen Balance, Milk Nitrogen, Etc.
Animal 1. Grade Guernsey.

Date.	Nitrogen.					
	Intake.	Feces.	Absorbed.	Urine.	Milk.	Balance.
Gluten feed period.						
	gm.	gm.	gm.	gm.	gm.	gm.
Dec. 11–17	1,101	483	618	407	436	−225
" 18–24	1,101	429	672	493	405	−224
" 25–31	1,101	461	640	342	420	−122
Jan. 1–7	1,101	452	649	307	397	− 55
Oil meal period.						
Jan. 8–14	1,101	437	664	406	400	−142
" 15–21	1,101	430	671	426	382	−137
" 22–28	1,101	401	700	414	372	− 86
" 29–Feb. 4	1,101	415	686	393	371	− 78
Distillers' grains (Ajax) period.						
Feb. 5–11	1,101	498	603	294	385	− 76
" 12–18	1,101	506	595	257	376	− 38
" 19–25	1,101	513	588	282	397	− 91
" 26–Mar. 4	1,101	558	553	293	406	−156
Cottonseed meal period.						
Mar. 5–11	1,101	562	539	325	407	−193
" 12–18	1,101	604	497	357	371	−231
" 19–25	1,101	547	554	349	393	−188

duction. The production energy of the ration fed conformed closely to Armsby's standard of 7 therms daily for maintenance and 0.3 therm for each pound of 3 per cent milk produced by a 1,000 pound cow. For example, Animal 3 weighed 1,000 pounds and produced daily 20 to 24 pounds of milk containing 4.5 to 5 per

cent of fat. It consumed 38 pounds of the ration, equivalent to 15.5 production therms. In 50 pounds of any of these rations there were approximately 2.32 pounds of digestible protein, 37 per cent of which came from the particular concentrate under investigation.

TABLE IV.

Record of Nitrogen Balance, Milk Nitrogen, Etc.
Animal 2. Pure Bred Jersey.

Date.	Nitrogen.					
	Intake.	Feces.	Absorbed.	Urine.	Milk.	Balance.
Gluten feed period.						
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Dec. 4–10	1,296	636	660	383	478	−201
" 11–17	1,296	598	688	424	451	−177
" 18–24	1,296	574	722	386	454	−118
" 25–31	1,296	585	711	373	444	−106
Oil meal period.						
Jan. 1– 7	1,296	569	727	391	480	−144
" 8–14	1,296	557	739	403	469	−133
" 15–21	1,296	520	776	429	451	−104
" 22–28	1,296	504	792	384	435	− 27
Distillers' grains (Ajax) period.						
Jan. 29–Feb. 4	1,296	584	712	301	415	− 4
Feb. 5–11	1,296	627	669	327	423	− 81
" 12–18	1,296	624	672	310	427	− 65
" 19–25	1,296	661	635	326	438	−129
Cottonseed meal period.						
Feb. 26–Mar. 4	1,296	690	606	385	454	−233
Mar. 4–11	1,296	651	645	355	461	−171
" 12–18	1,296	669	627	358	438	−169
" 19–25	1,296	692	604	339	442	−177

In Tables III, IV, and V are recorded by weekly periods the nitrogen balances and the nitrogen secreted in the milk. The figures represent the intake and outgo for the total 7 days. Charts are also added showing the positive or negative nitrogen balances and the gm. of nitrogen secreted in the milk.

A survey of the data shows that there was considerable variation in nitrogen metabolism with the different protein mixtures. The gluten feed, oil meal, and cottonseed meal rations were somewhat similar in respect to the way their nitrogen was being used,

TABLE V.

Record of Nitrogen Balance, Milk Nitrogen, Etc.
Animal 3. Grade Guernsey.

Date.	Nitrogen.					
	Intake.	Feces.	Absorbed.	Urine.	Milk.	Balance.
Oil meal period.						
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Dec. 4–10	1,222	533	689	442	461	−234
" 11–17	1,231	475	756	411	446	−101
" 18–24	1,231	494	737	444	420	−127
" 25–31	1,231	552	679	395	428	−144
Gluten feed period.						
Jan. 1– 7	1,231	499	732	390	412	− 70
" 8–14	1,231	493	738	431	407	−100
" 15–21	1,231	502	729	429	381	− 81
" 22–28	1,231	449	782	450	366	− 34
Distillers' grains period.						
Jan. 29–Feb. 4	1,231	593	638	370	363	− 95
Feb. 5–11	1,231	595	636	303	368	− 35
" 12–18	1,231	594	637	326	363	− 52
" 19–25	1,231	576	655	287	373	− 5
Cottonseed meal period.						
Feb. 26–Mar. 4	1,231	675	556	408	387	−239
Mar. 4–11	1,231	657	574	397	375	−198
" 12–18	1,231	607	624	412	368	−156
" 19–25	1,231	688	543	429	365	−251

although with all the animals and especially No. 3, there was a lower efficiency of protein utilization in the cottonseed meal period. All animals showed the greatest efficiency in protein utilization in the distillers' grains period, amounting by our method of calculation to 10 to 12 per cent above the other mixtures.

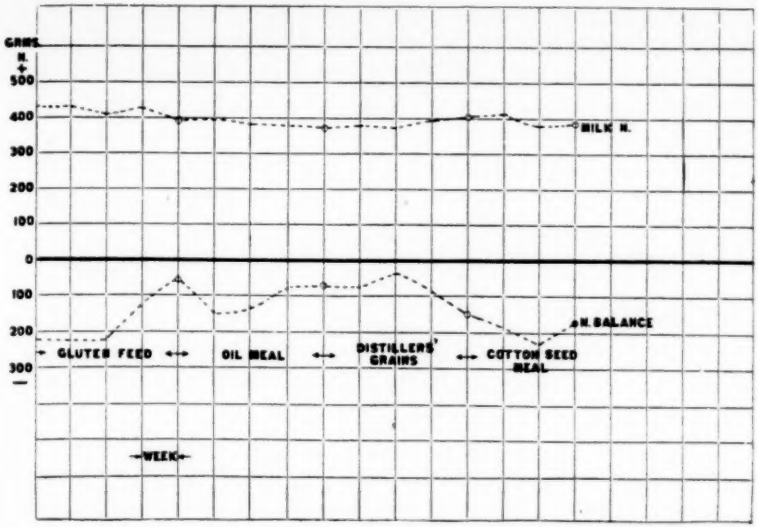

CHART 1. Animal 1. Showing the nitrogen balances with the different sources of protein and the milk nitrogen produced.

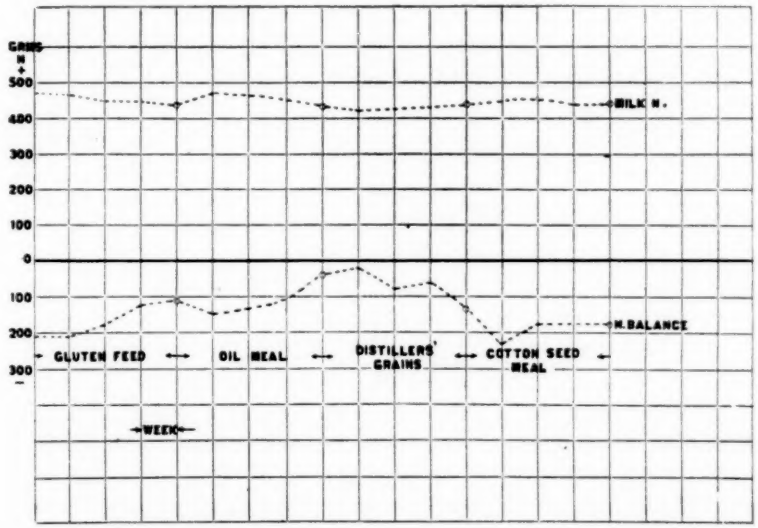

CHART 2. Animal 2. Showing the nitrogen balances with the different sources of protein and the milk nitrogen produced.

In our experiments with clover hay, reported in 1917, there was a tendency toward a more effective supplementary relation between clover hay and distillers' grains, but in these experiments with alfalfa hay the effect was more marked and unmistakable. The distillers' grains were the same as those used in our work in 1917 and were mainly derived from the corn grain. They would differ from gluten feed mainly in their content of the corn germ, to the proteins of which we attribute this superior supplementing effect.

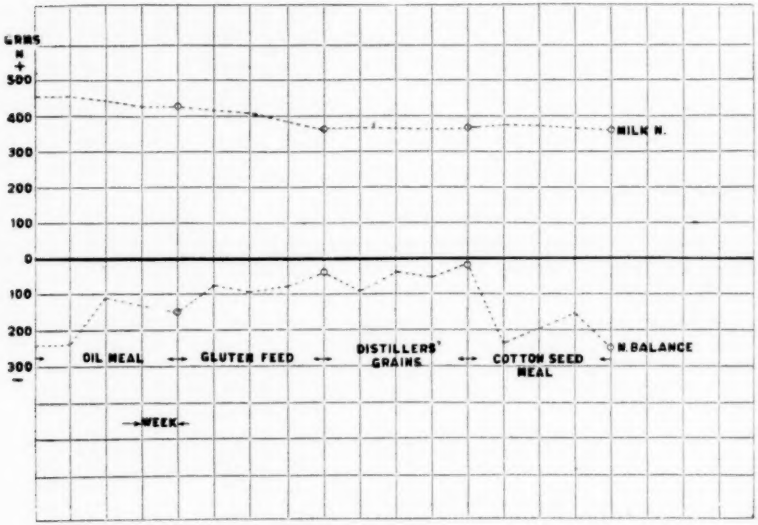

CHART 3. Animal 3. Showing the nitrogen balances with the different sources of protein and the milk nitrogen produced.

As distillers' grains are now off the market it would seem possible to provide dairymen with a protein concentrate of very high chemical efficiency, so far as its protein is concerned and where alfalfa or clover is the main roughage used, by combining gluten feed and corn germ meal. Since the germ constitutes about 10 per cent of the weight of the maize kernel, a mixture with that proportion, or possibly 15 per cent as germ oil meal, should be effective.

Accompanying an approach to nitrogen equilibrium when fed the distillers' grains ration there was also a marked decrease in

urinary nitrogen output during this period. This was shown by all animals as they pass from either the oil meal or gluten feed ration to the distillers' grain ration. When changed from the latter to the cottonseed meal ration there were marked increases in urinary nitrogen output, although the animals were maintained on the same nitrogen intake. These facts are further evidence of a more effective supplementing between the nitrogen of the distillers' grains and the nitrogen of the rest of the ration than in the case of any of the other concentrates.

These studies again confirm what we have already stated; namely, that there is a limitation to any classification of natural feeds or foods in respect to the efficiency of their proteins, based on the determination of such nutritive worth in a single food material or mixture. Gluten feed is less efficient as a source of protein when the roughage is corn stover than when it is clover or alfalfa hay. Maize meal proteins show poor quality for growth when used with gluten feed, a greater efficiency when mixed with oil meal, and a still greater efficiency when mixed with milk proteins.

These animals were in negative nitrogen balance during the entire 16 weeks, but approached nitrogen equilibrium during the distillers' grain ration period. But even with a continuous negative nitrogen balance their live weight was maintained. Their initial weights were 830, 800, and 968 pounds respectively. At the termination of the experiment they weighed 831, 830, and 1,025 pounds respectively. In spite of this apparent gain in weight the animals were plainly becoming more and more emaciated. This condition was especially noticeable in the region of the femoral muscles where a marked wasting was evident. The gains in weights may have been due to water replacement or fat, as ample therms were provided. In spite of a continued negative nitrogen balance the flow of milk was remarkably sustained. There was a slow and gradual shrinkage in volume, more with some individuals than with others. Animal 1 was producing 23.2 pounds of milk at the beginning of the experiment and 18.5 at the termination; No. 2, 28.3 pounds at the beginning and 25.1 at the end; No. 3, 24.9 at the beginning and 17.8 at the end.

Through a continued negative nitrogen balance of 16 weeks Animal 2 lost 29.4 pounds of protein. If we accept 16 as

approximately the per cent of protein in this animal, as found for a half-fat ox at the Rothamsted Station, this cow, weighing 800 pounds, would have contained 128 pounds of protein; she lost during these 16 weeks of observation 23 per cent of her protein content and yet maintained a remarkable milk flow.

Those who have studied the salmon in its migration without food in fresh water have been inclined to believe that this fish starts the journey with a superload of protein in the muscle cells. Greene[5] records the fact that the muscle proteins of this fish are diminished by 30 per cent during the migration without affecting

TABLE VI.

Decrease in Milk Volume, but Maintenance of Percentage Composition of Milk with Cows in Negative Nitrogen Balance.

Animal.		Dec. 17	Jan. 14	Feb. 11	Mar. 18
1	Total solids, *per cent*	13.07	14.20	14.20	15.30
	Fat, *per cent*	4.5	5.25	5.10	5.30
	Nitrogen, *per cent*	0.61	0.60	0.61	0.68
	Milk daily, *lbs*	22.00	20.70	19.80	16.70
2	Total solids, *per cent*	12.12	12.42	12.80	13.52
	Fat, *per cent*	4.15	4.10	4.15	4.40
	Nitrogen, *per cent*	0.50	0.57	0.53	0.60
	Milk daily, *lbs*	28.00	26.40	25.10	21.40
3	Total solids, *per cent*	12.77	13.30	13.65	14.31
	Fat, *per cent*	4.50	4.57	4.40	4.90
	Nitrogen, *per cent*	0.58	0.60	0.59	0.63
	Milk daily, *lbs*	24.20	21.30	19.80	17.80

the physiological or histological characteristics of the tissue. In the case of this fish, under the stimulus of reproduction, the muscles supply the material for ovary building. Under the stimulus of milk production and with a deficient protein supply, protein losses to the same degree may take place without exhausting the animal; but there was probably in this case no superload of proteins in the muscle cells as a necessary condition for sustained milk production. These animals were all in only ordinary flesh when the experiment was begun.

[5] Greene, C. W., *J. Biol. Chem.*, 1918, xxxiii, p. xiii.

While there was a shrinkage in the volume of milk produced and consequently a decrease in the absolute quantity of solids elaborated by these animals, there was no decrease in the percentage composition of the milk. Confirmation of this statement will be found in Table VI. With certain individuals under prolonged negative nitrogen balance there will be a gradual decrease in the percentage composition of the milk,[6] but there was no evidence of such a condition with any of these individuals. We would, therefore, be in error if we should attempt to postulate as a rule that all milking animals under negative nitrogen balance would show a decrease in the percentage composition of their milk.

Efficiency of Protein Mixtures Compared.

Individual variation, due to temperament, status of nutrition, etc., is such an important factor in the milking capacity of animals and their use of feed that we should avoid attaching absolute rigidity to the values of the efficiency of protein mixtures which we have previously submitted and will submit in this paper. There is a degree of difference in this efficiency which can with safety be recognized for different protein mixtures used on the same animal, but these same mixtures may not give exactly the same value with another individual, although the second individual should, of course, show approximately the same kind of response. We would consider a corn stover-corn grain-gluten feed protein mixture always inferior to a clover hay-corn grain-gluten feed mixture for milk production, but two animals may differ materially in the numerical expression of this efficiency.

The basis for the comparison of these protein mixtures involves not only the milk proteins elaborated, but in addition the protein catabolized or stored during the periods of observation. *It is an expression of the value of the proteins of the mixture for maintenance, growth, and milk production.* It could hardly be considered an expression for maintenance and milk production alone because of the fact that these animals, while maintaining their live weight, were in negative nitrogen balance and not completely maintaining their tissue nitrogen. What restoration of protein tissue was made could probably, with justification, be considered as growth.

[6] Hart and Humphrey, *J. Biol. Chem.*, 1916, xxvi, 457.

For purposes of making comparisons we have calculated the per cent of efficiency for the various mixtures on the basis of absorbed nitrogen rather than on the total nitrogen ingested. It is true that in our growth experiments with swine,[3] comparisons of protein efficiency have been made on the basis of absorbed as well as total ingested nitrogen and such experiments have shown an efficiency based on the total ingested nitrogen of from approximately 25 per cent for the cereal grain proteins to 65 per cent for milk proteins. On the basis of absorbed nitrogen, using swine and concentrated materials, the figures differ but little from those secured by a calculation based on the total nitrogen ingested. By the same method of calculation, that is by the use of the *total* ingested nitrogen rather than the absorbed nitrogen, the efficiency of the corn stover-grain protein mixture used in 1916 was as low as 22 per cent for the corn stover-gluten feed mixture and as high as 40 per cent for a corn stover-oil meal ration. The clover hay-grain protein mixture used in 1917 showed variations in efficiency, dependent upon individuals and mixture, of from 25 to 35 per cent when based on the *total* nitrogen ingested and from 50 to 75 per cent when based on the total *absorbed* nitrogen. In any case there is no general approach in the efficiency of these mixtures to those of milk proteins used alone with swine. Even in the case where we used milk powder and corn stover as one of the mixtures for comparative studies on milk production, the efficiency was but 34 per cent based on the total ingested nitrogen and 60 per cent on the basis of the absorbed nitrogen. The latter figure compares well with results secured with swine by the same method of calculation. In work with herbivora, requiring a roughage of great bulk, the fecal nitrogen as an excretory factor is so large that the balance left for the productive processes is greatly reduced as compared with experiments on omnivora receiving concentrates only, where the fecal nitrogen output is relatively small.

For these reasons it would appear more correct to base the calculation of the efficiency of protein mixtures for all classes of animals on the absorbed nitrogen rather than on the total nitrogen ingested; but it must also be apparent that any condition which increases the fecal nitrogen output, as for example, ingestion of abundant roughage, would increase the total nitrogen losses to the animal. In fact with omnivora, as swine, consuming abun-

dant roughage, the loss of fecal nitrogen is so large that unless special provision is made for the intake of a high plane of efficient protein concentrates the protein ingested cannot meet the growth impulse and rapid gains in weight will be impossible.

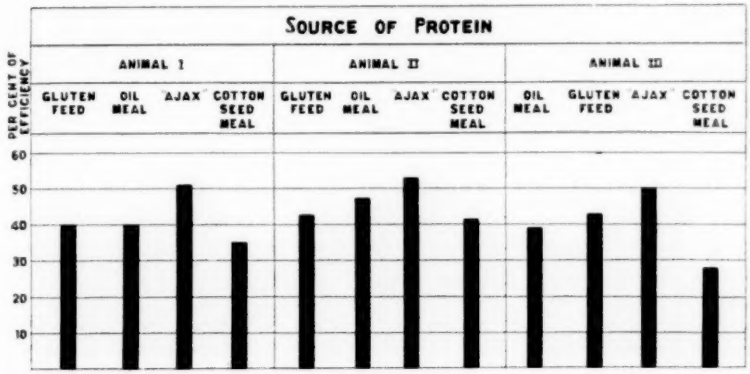

CHART 4. Showing the comparative efficiency for milk production of the different protein mixtures. The chart shows that there was a general agreement among the different individuals, but a difference in the effect of the mixtures.

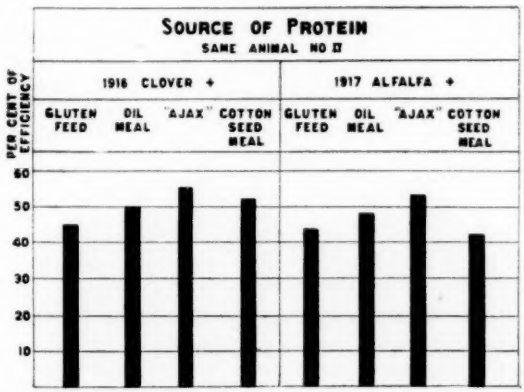

CHART 5. This chart illustrates the close agreement in the efficiency of protein utilization by the same individual in 2 succeeding years. The rations differed only that in 1916 clover was used while in 1917 alfalfa was used.

In Table VII and Chart 4 comparisons of the different protein mixtures by individuals are presented. Chart 5 is added, comparing the utilization of protein mixtures by the same individual in two different years. In this latter case there was a fairly close agreement between the protein utilizations, with the exception of the cottonseed meal period and alfalfa hay compared with the same concentrate and clover hay. The former mixture was less efficient than the latter.

TABLE VII.

Relative Efficiency for Milk Production of Protein Mixtures Involving Alfalfa Hay, Gluten Feed, Distillers' Grains, Oil Meal, and Cottonseed Meal.

Animal.	Date.	Ration.	N absorbed.	N in milk + tissue formed or destroyed.	Efficiency.
			gm.	*gm.*	*per cent*
1	Dec. 11–Jan. 7	Gluten feed.	2,579	1,032	40
	Jan. 8–Feb. 4	Oil meal.	2,721	1,082	40
	Feb. 5–Mar. 4	Distillers' grain.	2,339	1,203	51
	Mar. 5– " 25	Cottonseed meal.	1,590	559	35
2	Dec. 4–Dec. 31	Gluten feed.	2,781	1,225	43
	Jan. 1–Jan. 28	Oil meal.	3,034	1,427	47
	" 29–Feb. 25	Distillers' grain.	2,688	1,424	53
	Feb. 26–Mar. 25	Cottonseed meal.	2,482	1,045	42
3	Dec. 4–Dec. 31	Oil meal.	2,861	1,149	39
	Jan. 1–Jan. 28	Gluten feed.	2,981	1,281	43
	" 29–Feb. 25	Distillers' grain.	2,566	1,280	50
	Feb. 26–Mar. 25	Cottonseed meal.	2,297	651	28

SUMMARY.

Data are presented on the comparative value for milk production of protein mixtures involving *alfalfa hay* and gluten feed, oil meal, distillers' grains, or cottonseed meal.

The concentrates furnished approximately 37 per cent of the digestible proteins of the ration and were used to supplement a basal ration of corn-meal, corn silage, and alfalfa hay. The total protein intake constituted about 10 per cent of the dry matter of the ration and the nutritive ratio was approximately 1:8.4. On

this low protein intake and with fairly liberal milk secretion negative nitrogen balances were maintained during the entire period of observation (16 weeks) with a slow shrinkage in milk volume, but with a striking maintenance of the percentage composition of the milk.

Earlier records showed the inferiority for milk production of the proteins of gluten feed to those of oil meal or distillers' grains as a supplement to the proteins of *corn stover*—corn-meal. Later records showed an equality in efficiency between the proteins of the gluten feed, oil meal, distillers' grains, and cottonseed meal as supplements to the proteins of corn-meal and *clover hay* for milk production; these records show an approximate equality and efficiency of the proteins of gluten feed and oil meal as supplements to the proteins of corn-meal and *alfalfa hay*, an increased supplementing effect for distillers' grains, and an inferior efficiency for cottonseed meal.

That the proteins of distillers' grains were more effective than those of gluten feed is due very probably to the fact that the former contains the embryo proteins. In view of the fact that for the present distillers' grains are not obtainable, these results suggest the possibility of making an equally efficient protein mixture by adding 10 to 15 per cent of germ oil meal to gluten feed.

These results again emphasize the limitations of any classification of natural foods in respect to the efficiency of their proteins based on the determination of such nutritive worth in a single food material or a single food mixture.

A METHOD FOR THE SEPARATION AND QUANTITATIVE DETERMINATION OF THE LOWER ALKYLAMINES IN THE PRESENCE OF AMMONIA.*

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It is well recognized that the amines, as well as ammonia, are products of protein decomposition. Allen[1] quotes several authors who report finding one or more of the alkylamines in decomposed protein products. Thus Bocklisch[2] frequently found methylamine in spoiled fish. He suggests that it is formed in such material by the loss of carbon dioxide from glycine and also from trimethylamine. Dimethylamine was found in herring brine by Bocklisch, in putrid gelatin and yeast by Brieger, and in poisonous sausage by Ehrenberg.[3] In the bacterial cultures from this sausage Ehrenberg reports finding diethylamine.

Allen also states that ethylamine has been obtained from putrid yeast and flour, and that trimethylamine is a common putrefactive product found in herring brine and in extract of ergot.

It is known that various forms of amines occur in the tissues of fish. In the preparation of salt-cured fish, amines are extracted or formed from the fish proteins, herring brine, for example, furnishing a commercial source for trimethylamine.

In the determination of ammonia in biological fluids and in food products by Nesslerizing[4] the volatilized base, obtained by aera-

* Published with the permission of the Secretary of Agriculture.

[1] Allen, A. H., Commercial organic analysis, Philadelphia, 4th edition, 1913, vii, 345, 352.

[2] Bocklisch, O., *Ber. chem. Ges.*, 1885, xviii, 1922.

[3] Ehrenberg, A., *Z. physiol. Chem.*, 1887, xi, 239.

[4] Folin, O., and Macallum, A. B., *J. Biol. Chem.*, 1912, xi, 523.

tion of the sample, erroneous results may be obtained if the volatile alkaline material is composed, in part, of alkylamines.

The determination of the total volatile alkaline material when both ammonia and alkylamines are present can be effected by titration, using dilute standardized solutions. This procedure does not distinguish between the different bases which may be present, the results being calculated as ammonia.

In case of fish products the ammonia determination, by titration of the volatilized alkaline material, yields results including both ammonia and amines. The determination of ammonia by the

TABLE I.

Determination of Ammonia in Fish by the Nesslerization and Titration Methods.

	"Ammonia" nitrogen per 100 gm. (moisture fat-free basis).	
	Nesslerization.	Titration.
	mg.	*mg.*
Fish before canning. In salt 4 hrs.	67.3*	73.6*
" " " " " 6 "	52.2*	61.4*
Canned fresh fish. No salt or pickle	107.8	147.6
" fish 24 hrs. old. No salt or pickle	121.7	189.6
" fresh fish, same as above, after standing 7 mos.	108.7	210.2
" fish 24 hrs. old, same as above, " 7 "	119.6	227.2

* Also on salt-free basis.

Nesslerizing method in the total volatile alkaline material is inaccurate because of the presence of amines.

In the investigations conducted in the Maine sardine industry, by this laboratory, a number of determinations of "ammonia" by both the Nesslerization and titration methods were made on samples of canned sardines. In some instances the quantity of "ammonia" found where both methods of analysis were applied agreed quite closely, but in the majority of cases there was a very wide variation in the amounts determined by the two methods. A few of the determinations, typical of this discrepancy, are given in Table I.

The need of a method for the separation of ammonia and amines arose in connection with the analytical work in this investigation

and in the analyses made on other fish products. It was also desirable in connection with this work to find a method by which the three classes of amines, the primary, secondary, and tertiary forms, might be separated from one another and their quantity accurately determined.

A number of methods were found in the current literature which were especially designed to purify each of the amines by the separation of the one from the other and from ammonia. Methods of this general type were not suited to the exact determination of these compounds. In most of those cases where the method of separation was exact, or where it was believed to be upon a good basis or capable of refinement to render it accurate, such a large quantity of material (amines) was required as to make a method which might be developed impractical for use where small quantities of ammonia and amines were to be determined.

EXPERIMENTAL.

A few methods were found in the rather copious literature bearing upon the separation and determination of the amines, which it was thought should be investigated in order to test their accuracy and to determine whether they would meet the requirement sought; namely, a complete separation and accurate quantitative determination of small quantities of ammonia and amines.

The amine solutions employed in these experiments were all prepared from Kahlbaum's reagents; both the 33 per cent solutions and the salts of the different amines as the hydrochlorides were used.[5] The ammonia used was the ordinary "ammonium hydroxide" reagent of Baker and Adamson. Solutions of 0.1 N and 0.05 N strength were prepared from the above reagents for use in the experiments.

Separation of Total Amines from Ammonia.

In this study of the available methods that of Budai[6] was first tried. The separation of the ammonia is dependent, in this

[5] Their purity was tested for us by Dr. E. T. Wherry, Crystallographer of the Bureau of Chemistry, and found to be more than 99 per cent pure. Additional evidence of their purity is shown by the close agreement of results obtained by direct titration and after separation by the method proposed.

[6] Budai (Bauer), K., *Z. physiol. Chem.*, 1913, lxxxvi, 107, 121.

method, upon the formation of aldehyde-ammonia, using a neutral (to phenolphthalein) solution of formaldehyde for this purpose.

The method as it was applied to known solutions follows. The solution containing the total volatile base, ammonia and amines, was made neutral to methyl red with a dilute solution of potassium hydroxide. 10 cc. of 30 per cent formaldehyde solution, made neutral to phenolphthalein, were then added, and the mixture was allowed to stand for a few minutes. The solution was then titrated to a permanent pink color of phenolphthalein with 0.05 N potassium hydroxide. The exact amount of ammonia present was determined by the results of this titration. If it is desired, the solution may now be acidified with hydrochloric acid, concentrated by boiling to one-third its volume, and distilled by diluting to 200 cc. and making alkaline with 10 per cent sodium hydroxide. The distillate contains the total volatile base which may now be determined by direct titration, or, if already known, the titration may be made as a check on that determination.

The results obtained, by this method, using ammonia and amine solution of known strength and mixtures of these solutions, are given in Table II.

It was found that the Budai method gave accurate results for the separation of ammonia and trimethylamine, but failed when mono- or diamine was added.

A method proposed by Quantin[7] was then tried. This method is based upon the fact that in alkaline solution ammonia will precipitate magnesium and phosphoric acid as magnesium ammonium phosphate whereas the amines present do not form a precipitate.

It was found that if the precipitated magnesium ammonium phosphate was dissolved in hydrochloric acid, treated with strong caustic soda and distilled, the ammonia was recovered completely. Portions of pure magnesium ammonium phosphate, prepared in the laboratory, were weighed, dissolved in hydrochloric acid, made alkaline with sodium hydroxide, and distilled into 0.05 N sulfuric acid. The results obtained on the pure salt with this method of treatment are shown in the following statement.

[7] Quantin, H., *Ann. chim. anal. appl.*, 1901, vi, 125.

Weight of salt, *gm*	0.4000	0.3500	0.2000	0.3000	
Nitrogen recovered, *gm*	0.02282	0.01981	0.01138	0.01705	
Nitrogen recovered, *per cent*	5.705	5.660	5.688	5.682	
Average recovered, *per cent*					5.684

TABLE II.

Results Obtained by the Use of Formaldehyde for the Separation of Ammonia and Amines (Budai's Method).

Solution mixtures.	Amine present (0.05 N).	Ammonia (0.05 N).	
		Present.	Found.
	cc.	cc.	cc.
Ammonia		25.57	25.47
"		20.00	20.04
"		27.75	27.82
"		29.75	29.75
Methylamine	24.67		24.11
Dimethylamine	25.60		14.51
Trimethylamine	16.17		1.30
"	26.18		0.94
Methylamine and ammonia	10.04	15.12	23.18
" " "	16.42	8.75	23.80 23.38 24.12
Dimethylamine and ammonia	16.83	8.42	15.97
" " "	9.82	15.70	20.78
Trimethylamine and ammonia	19.04	5.00	5.48
" " "	3.80	20.00	19.92
" " "	3.79	20.15	19.84
" " "	13.96	12.20	12.63
" " "	9.42	15.00	15.19
" " "	6.74	19.20	19.26
" " "	0.84	22.90	22.81
Methylamine Dimethylamine and ammonia	9.96 10.08	8.06	20.44

The theoretical amount of nitrogen in magnesium ammonium phosphate is 5.701 per cent, therefore 99.70 per cent of the total was recovered. In order to test the application of this reaction to the separation of ammonia and amines solutions of pure magnesium sulfate and sodium acid phosphate were prepared of such strength that when mixed in equal volumes 1 cc. was equivalent

to 1 cc. of 0.05 N ammonia. These two solutions contained 12.04 gm. of $MgSO_4.7H_2O$ per liter, and 35.84 gm. of $Na_2HPO_4.12H_2O$ per liter.

The ammonia or amine solution was neutralized with 0.05 N sulfuric acid, 10 cc. of each of the above solutions were added, and after thorough shaking the mixture was allowed to stand for a short period of time. It was then filtered, the precipitate containing the ammonia, as magnesium ammonium phosphate, was transferred to a distilling flask and dissolved in a few cc. of hydrochloric acid. The solution was then diluted to 200 cc., made alkaline by the addition of 10 cc. of 10 per cent sodium hydroxide, and distilled into 0.05 N sulfuric acid. The results obtained follow.

Present.	Recovered as 0.05N ammonia.
	cc.
Ammonia equivalent to 19.85 cc. 0.05 N	9.00
" " " 19.85 " 0.05 "	18.00
Ammonia " " 9.93 " 0.05 " / Monoamine " " 9.90 " 0.05 "	9.20
Ammonia " " 9.93 " 0.05 " / Diamine " " 9.97 " 0.05 "	7.10
Monoamine " " 19.80 " 0.05 "	0.70

The next determinations were made in the same way except that 25 cc. of the precipitating solutions were used and the solutions before precipitation were made alkaline with potassium hydroxide, monomethylamine, and caustic soda, in amounts indicated in Table III, which gives the results obtained by this variation in the procedure.

These data show that the separation of the amines from ammonia is not complete under the conditions enumerated. In the original method Quantin uses monomethylamine to make the solution alkaline for the precipitation of magnesium ammonium phosphate. This is objectionable because in our work it was desired to determine monomethylamine. With the small amount of alkaline volatile material which is likely to be found in ordinary food or biological products it is undesirable to make this determination on a separate quantity. In one of the above experiments, when ammonia and monomethylamine were treated in the presence of potassium hydroxide, about 95 per cent of the ammonia was found in the precipitate and all of the monoamine

in the filtrate, but in another experiment in which monomethylamine was added in large excess to make the solution alkaline a large amount of it was found with the ammonia in the precipitate.

The method of Erdmann[8] which had previously been employed in routine work for this separation was next thoroughly investigated. This method depends upon the François[9] reaction in which the ammonia is absorbed by yellow mercuric oxide, whereas the amines are not so absorbed. It was found that the fineness

TABLE III.

Precipitation as Magnesium Ammonium Phosphate.

Present, equivalent to 0.05 N.		Made alkaline with	Recovered.	
			Ammonia (0.05 N).	Unprecipitated (amine) (0.05 N).
	cc.		cc.	cc.
Ammonia	19.80	25 cc. 0.05 N KOH	17.40	0.04
Monoamine	19.75	25 " 0.05 " KOH	11.80	8.00
Diamine	19.60	25 " 0.05 " KOH	1.45	18.00
Ammonia	9.90	25 " 0.05 " KOH	9.40	9.90
Monoamine	9.80			
"	19.75	20 " 0.05 " KOH	1.00	
Ammonia	19.60	20 " 0.05 " KOH	17.20	
"	9.90	20 " 0.05 " KOH	8.60	
"	20.00	10 " 0.1 " monoamine.	19.10	
"	9.90	30 " 0.1 " "	25.05	
"	10.00	10 " 10 per cent NaOH	0.05	

of the particles of the oxide have a marked influence on the accuracy of the separation. Yellow oxide of mercury which was in a very fine and amorphous state gave the best results. Merck's yellow oxide of mercury, labeled "Reagent-mercury oxide yellow Merck-mercuric," was used throughout these experiments and proved very satisfactory. The method employed was the following.

[8] Erdmann, C. C., *J. Biol. Chem.*, 1910, viii, 41.

[9] François, M., *J. pharm. et chim.*, 1907, xxv, 517; *Compt. rend. Acad.*, 1907, exliv, 857.

Separation of Ammonia and Amines by Use of Yellow Oxide of Mercury.

The solution, containing nitrogen as ammonia and amines (organic base) equivalent to 20 to 30 cc. of 0.1 N acid (the titer of the solution being previously determined), was diluted nearly to the mark in a 500 cc. graduated flask. 10 cc. of a solution containing

TABLE IV.

Results Obtained by Use of Yellow Oxide of Mercury for Separation of Ammonia and Amines (Erdmann's Method).

Solution mixtures.	Present as 0.05 N.		Found as 0.05 N amines.
	Ammonia.	Amines.	
	cc.	cc.	cc.
Ammonia	20.45		0.35
"	21.52		0.41
Methylamine		19.73	19.60
Dimethylamine		20.48	18.77
Trimethylamine		19.40	18.97
"		20.94	20.71
Methylamine and ammonia	12.00	8.03	7.56
Dimethylamine " "	12.66	7.86	7.47
Trimethylamine " "	4.00	15.23	15.04
" " "	16.00	3.04	3.42
" " "	16.12	3.03	3.40
" " "	9.76	11.17	11.23
" " "	12.00	7.54	7.69
" " "	15.86	5.39	5.81
" " "	19.20	0.67	1.30
Methylamine, dimethylamine, and ammonia.	6.45	7.97 8.06	14.81

equal parts of 20 per cent sodium hydroxide and 30 per cent sodium carbonate were then added. The solution was made up to the mark with water. An amount of yellow mercuric oxide reagent, determined by taking 0.1 gm. for each cc. of 0.1 N acid, to which the solution in the flask is equivalent, was now added. The flask was tightly corked, wrapped in a black cloth, because light is said to vitiate the results, and shaken for 1 hour. After standing over night to allow the mercuric oxide and the precipitate formed to

settle, the liquid was separated from the precipitate and excess of oxide by forcing the solution through a cotton filter by means of a moderate air pressure. 200 or 250 cc. were distilled into a measured amount of standardized acid, and the excess was titrated with 0.05 N potassium or sodium hydroxide.

TABLE V.

Results Obtained in Treatment of Amine Solutions by the Erdmann Method (Use of Yellow Oxide of Mercury).

500 cc. solution contained as 0.05 N.				In fraction distilled as 0.05 N.	
Monoamine.	Diamine.	Triamine.	Total amines.	Present.	Found.
cc.	cc.	cc.	cc.	cc.	cc.
51.87	—	—	51.87	20.75	20.65
51.87	—	—	51.87	20.75	20.50
47.70	—	—	47.70	19.08	19.00
—	51.50	—	51.50	20.60	20.60
—	51.50	—	51.50	20.60	20.60
—	47.83	—	47.83	18.95	18.84
—	—	42.90	42.90	17.18	17.23
—	—	46.75	46.75	18.70	18.55
20.75	31.05	—	51.80	20.70	20.50
20.75	—	28.05	48.80	19.52	19.40
—	20.70	28.05	48.75	19.50	19.25
20.75	20.70	9.35	50.80	20.32	20.60
10.40	10.35	28.05	48.80	19.52	19.50
23.80	9.47	17.18	50.45	20.18	20.18
19.08	23.68	8.59	51.35	20.54	20.35
9.54	18.95	21.47	49.96	19.98	19.78
25.39	12.61	18.49	56.49	22.59	22.22
20.31	25.21	11.43	56.95	22.78	22.75
12.70	20.17	22.86	55.73	22.29	22.22

The results obtained by this method when applied to mixtures of ammonia and the different amines are given in Tables IV and V.

As additional evidence that this reaction was complete, certain samples, containing no monoamine, were run in the Van Slyke amino-acid apparatus to find traces of ammonia. Table VI shows that no ammonia within the limits of error was present in solutions after the treatment with oxide of mercury.

TABLE VI.

Ammonia Nitrogen Found in Distillates from Mercuric Oxide Treatment.

Volume of gas as nitrogen.		
Determination.	Blank.	Ammonia.
cc.	*cc.*	*cc.*
1.04	1.06	−0.02
1.10		0.04
1.09	1.08	0.01
1.10		0.02
1.10	1.08	0.02
1.06		−0.02
1.08	1.08	0.00
1.14		0.06
1.14	1.12	0.02
1.11	1.10	0.01
1.11	1.10	0.00
1.10	1.10	0.00
1.12		0.02
1.08	1.10	−0.02
1.08		−0.02
1.03	1.04	−0.01
1.12	1.04*	0.08
1.04	1.04	0.00
1.04		0.00
1.02	1.04	−0.02
1.16	1.04*	0.12

* At a temperature 3° higher than the blank.

Separation and Determination of Primary, Secondary, and Tertiary Amines.

A number of methods have been suggested for the separation and determination of the three classes of amines, but most of them are unsatisfactory because they were originally designed for the puri-

fication of one amine by the elimination of the other two, rather than for the separation of these compounds in such a manner as to lead to the quantitative determination. Several of the promising methods found in the literature were investigated to determine their applicability and accuracy.

According to Bertheaume[10] the solubilities of the chloroplatinates of dimethylamine and trimethylamine in alcohol of various strengths are as follows:

At 0°, 100 gm. dissolve.

Alcohol, *per cent*	100	90	80	70	60
Diamine, chloroplatinate, *gm*	0.0048	0.110	0.325	0.558	0.996
Triamine, chloroplatinate, *gm*	0.0036	0.070	0.243	0.391	0.766

According to Seidell,[11] the solubility of ammonium chloroplatinate in water is 0.666 per 100 parts at 10° C., and 1.25 at 100°C. At 15°C. the solubility in alcohol of various strengths is:

Alcohol, *per cent*	55	76	95
Ammonium chloroplatinate	0.150	0.067	0.0037 parts per 100

These differences in solubility are not sufficiently marked to base an accurate method of separation upon them. A series of experiments were made, based upon the solubility of the platinum salts in alcohol. This proved to be unsatisfactory as a means of separation. A method for the determination of amines suggested by Bertheaume[12] was not tried because of the small amount of volatile alkaline material usually found in determinations made upon food products. This method depends upon the separation of the hydrochlorides of ammonia and monomethylamine from those of di- and trimethylamine, by the solution of the latter in chloroform. In this solvent the former compounds are insoluble. Ammonia is then separated from monomethylamine by the François reaction.[9] Trimethylamine is precipitated from the dissolved residue from the chloroform solution as the periodide. The periodides are soluble in solutions of sodium chloride, potassium chloride, and magnesium sulfate as follows: Diamine periodide

[10] Bertheaume, J., *Compt. rend. Acad.*, 1910, cl, 1063.

[11] Seidell, A., Solubilities of inorganic and organic substances, New York, 1907.

[12] Bertheaume, *Compt. rend. Acad.*, 1910, cl, 1251.

gives no precipitate when present in the dilution of 1:1,000; triamine periodide gives a precipitate at the dilution of 1:100,000.

The method of Muller[13] and that of Bresler[14] as well as that of Quantin[7] are inaccurate as they depend upon the different solubilities of the chloroplatinates of the amines in alcohol.

Seiler and Verda[15] recommend the use of phosphomolybdic acid for the precipitation of substances containing an amino group. In different classes of amino nitrogen compounds different colored precipitates result which may be reduced, resulting in another color. These colors are characteristic. The following results were obtained in a series of experiments based upon this method.

A solution of phosphomolybdic acid was prepared by dissolving 15 gm. in 200 cc. of water and 4 cc. of concentrated nitric acid. This solution was filtered and in each determination 10 cc. were used. To the solution of the amine in about 50 cc. of water, heated to 80°C., 10 cc. of the phosphomolybdic acid solution were added, and the mixture was shaken for a few minutes. As the solution cooled, a yellow precipitate began to settle out. The precipitation occurred first in the solution containing triamine, later in that containing diamine. No precipitate formed in the case of ammonia or monomethylamine, even after the solution remained at room temperature for some time (Table VII).

The results obtained by this procedure show that ammonia and monoamine do not precipitate, under the conditions, as the phosphomolybdates. The di- and triamines are precipitated but not quantitatively.

Delépine[16] proposed a method for the separation of the methylamines, which depends upon the difference in the boiling points of the condensation products of the primary and secondary amines with formaldehyde. The tertiary amines remain unchanged. No experiments were made with this method to determine whether or not it could be made quantitative when applied to small quantities of the amines.

In all of the methods that were tried, none was found that combined accuracy and adaptability to the small quantities of amines

[13] Muller, M. A., *Bull. Soc. chim.*, 1884, xlii, 202.
[14] Bresler, M., *Deutsch. Zuckerind.*, 1900, xxv, 1593.
[15] Seiler, F., and Verda, A., *Chem. Ztng.*, 1903, xxvii, 1121.
[16] Delépine, M., *Compt. rend. Acad.*, 1896, cxxii, 1064.

that were known to be present in the total volatile alkaline material obtained in the aeration method for ammonia in certain food products. It was thought that the reactions of the amines with nitrous acid might prove valuable as the basis of a method for the separation and determination of the amines. These reactions were next studied.

In order to purify trimethylamine from dimethylamine Heintz[17] used nitrous acid. Under the proper conditions the diamine com-

TABLE VII.

Precipitation as Phosphomolybdates.

Material.	Nitrogen.		Weight of precipitate.
	Present.	Found.	
	mg.	*mg.*	*gm.*
Ammonia	14.0	0.00	0.00
Monoamine	14.5	0.00	0.00
Diamine	8.97	11.21	0.5240
		9.31	0.4350
Triamine	11.38	12.26	0.5865
		12.98	0.6210
		11.65	0.5575
"	13.02	11.84	0.5663
		11.08	0.5303
		11.33	0.5423

bines with nitrous acid to form nitrosoamine. It is known also that monoamine reacts with nitrous acid with the liberation of nitrogen. The reactions of the amines and ammonia with nitrous acid are as follows:

$$NH_3 + NOOH = 2H_2O + N_2$$
$$RNH_2 + NOOH = ROH + H_2O + N_2$$
$$RR'NH + NOOH = RR'NNO + H_2O$$
$$RR'R''N + NOOH = NRR'R''HOON$$

[17] Heintz, W., *Ann. Chem.*, 1866, cxxxviii, 319.

By means of nascent hydrogen obtained by the action of zinc and hydrochloric acid, the diaminenitrosoamine, obtained from the action of nitrous acid and diamine, can be reduced to diamine.

$$2\ RR'NNO + 4\ H = 2RR'NH + H_2O + N_2O$$

The Van Slyke amino-acid apparatus was first considered in connection with the determination of the nitrogen evolved from ammonia and monoamine. In the experiments made with this reaction in this apparatus it was found desirable to modify slightly the micro-apparatus employed in the method of Van Slyke.[18] The mixing bulb of the larger apparatus and the measuring burette of the micro-apparatus were combined in order to secure the greater accuracy of reading with the latter, and the larger size of sample permitted by the use of the former.

The time necessary for the complete evolution of nitrogen from the monoamine was first determined. A few experiments were also made to ascertain whether the di- and triamines would yield nitrogen at the same intervals of time during which the reaction with nitrous acid was allowed to progress. The time necessary for the complete reaction of ammonia in the Van Slyke apparatus was also determined. The results are given in Table VIII.

These results show that monoamine is completely decomposed and all of the nitrogen evolved is collected when the reaction is allowed to progress for 10 and 15 minutes. 5 minutes for shaking the apparatus appeared to be ample time to free and collect all of the nitrogen gas. 30 minutes is indicated as the time necessary for the reaction to continue to decompose the ammonia quantitatively. In the few determinations made in this experiment, no nitrogen was obtained when solutions of the di- and triamine were treated in the deaminizing bulb of the apparatus.

In order to be satisfied that no nitrogen was obtained in the case of the two higher amines and that the evolution of nitrogen was complete in the case of monoamine and ammonia, determinations were made on a number of samples of ammonia, monomethylamine, dimethylamine, trimethylamine, ethylamine, and diethylamine. The reactions, in all cases, were allowed to continue for 30 minutes and the apparatus was then shaken for 5 minutes. The results are given in Table IX.

[18] Van Slyke, D. D., *J. Biol. Chem.*, 1913–14, xvi, 121.

These results show that within the limits of error there is no nitrogen evolved in the case of the di- and triamines, and that in the case of monoamine and ammonia all of the nitrogen is found in the gas collected from the reaction with nitrous acid in the Van Slyke apparatus.

The experimental work on the separation of ammonia from the amines, by means of the François reaction, demonstrated that this separation could be accurately effected. Therefore, ammonia as a disturbing factor in the determination of monoamines could be

TABLE VIII.

Determination of Time Required for Complete Reaction of Ammonia, Mono-, Di-, and Triamine in the Van Slyke Amino-Acid Apparatus.

Standing.	Shaken.	Ammonia.		Monoamine.		Diamine.		Triamine.	
		Present.	Found.	Present.	Found.	Present.	Found.	Present.	Found.
min.	*min.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
5	5	3.36	2.69						
10	5	3.36	2.83	14.31	14.20 14.37 14.31	14.00	0.00 0.05	14.00	0.05 0.05
15	5	3.36	2.94	14.31	14.32	14.00	0.10	14.00	0.00
30	5	3.36	3.31			14.00	0.00	14.00	0.05
60	5	2.87	2.86 2.86	3.57	3.59				
180	5			3.57	3.59				

eliminated from consideration in the amine fraction. The nitrogen evolved from the reaction with nitrous acid and monomethylamine in the amine fraction consequently could be determined in the Van Slyke apparatus designed for the estimation of the aliphatic amino-acids.

Table X contains the results of determinations of different quantities of monoamine made when both di- and triamine were present in various proportions in the deaminizing bulb of the amino-acid apparatus. In this table the quantities of di- and

TABLE IX.

Results Obtained with the Van Slyke Apparatus.

	Nitrogen.	
	Present.*	Found.
	mg.	*mg.*
Ammonia	3.09	3.13
		3.24
		3.10
		3.11
	3.06	3.05
		3.10
		3.07
		3.05
		3.06
	1.53	1.40
		1.55
		1.56
Methylamine	3.55	3.49
		3.71
	2.96	2.98
		2.93
Ethylamine	2.82	2.81
		2.83
	2.85	2.91
		2.84
	2.82	2.83
		2.82
	2.85	2.85
		2.85
	1.48	1.45
	1.46	1.44

* By titration.

TABLE IX—*Concluded.*

	Nitrogen.	
	Present, approximately 0.1 N.	Found, gas.
	cc.	*cc.*
Dimethylamine	4.0	0.04
	10.0	0.00
		0.01
		0.03
		0.00
		0.00
		0.00
		0.00
		0.02
Diethylamine	10.0	0.06
		0.06
		0.00
		0.02
	2.0	0.05
		0.02
Trimethylamine	10.0	0.00
		0.04
		0.02
		0.00
		0.00
		0.04
		0.00
		0.00
		0.00
	4.0	0.02
		0.02

triamine are expressed in terms of cc. of 0.05 N solutions, and the amount of monoamine present and found is stated as mg. of nitrogen.

These results show that small quantities of the monoamine can be quite accurately determined in the presence of different amounts of the other two amines.

The reactions between di- and triamines with nitrous acid were next investigated. These two amines were brought into contact with nitrous acid which was generated from a saturated sodium nitrite solution and glacial acetic acid. It was found that known quantities of the triamines which had been acted upon by nitrous acid could be quantitatively recovered by distilling into standard-

TABLE X.

Determinations of Monoamine in the Presence of Di- and Triamine Using the Van Slyke Apparatus.

Present as 0.05 N.		Monoamine.	
Diamine.	Triamine.	Present.	Found
cc.	*cc.*	*mg.*	*mg.*
3.8	6.9	3.55	3.50 3.51
3.8	6.9	3.55	3.41 3.44
5.0	7.3	3.55	3.52
9.5	3.4	2.84	2.88 2.84
9.5	3.4	2.84	2.86
4.6	10.1	2.84	2.86
7.6	8.6	1.42	1.44 1.41
7.6	8.6	1.34	1.28 1.31
8.1	9.2	1.78	1.77

ized acid, after removal of the nitrous oxide and making the solution alkaline. The diamine nitrosoamine, it was also found, could be distilled without decomposing. It came over in the distillate with the triamine and could be quantitatively determined by reduction with nascent hydrogen and again distilling as diamine into standardized acid. The following experiments were made with

the di- and triamines to determine their deportment under the conditions as outlined.

The solution of the amines was added to 20 cc. of saturated sodium nitrite solution in a 200 cc. Erlenmeyer flask. 10 cc. of glacial acetic acid were then added. The flask was allowed to stand for $\frac{3}{4}$ to 1 hour, during which it was occasionally shaken. After standing, the flask was vigorously shaken for 5 to 10 minutes to expel as much of the oxides of nitrogen as possible. The solution was then nearly neutralized with sodium hydroxide, and after again standing an excess of caustic soda was added and the solution distilled into 0.05 N sulfuric acid. The triamine was determined in this distillate, which also contained the diamine in the form of nitrosoamine.

The diamine was recovered in the distillate by reducing the diamine nitrosoamine with nascent hydrogen. The distillate was made distinctly acid with 10 cc. of concentrated hydrochloric acid and a few gm. of granulated zinc were added. After boiling a few minutes, the solution was decanted from the remaining zinc, made alkaline with caustic soda, and the diamine distilled into standardized acid.

A large number of experiments were made in order to obtain the proper conditions regarding strengths of solutions used, and the time the different reactions should be allowed to proceed. That the basic principles of the method proposed were correct is seen by the results obtained in Table XI. The different amines listed therein were subjected to the treatment outlined above. The distillates were titrated each time for the respective amines, with the results as shown in Table XI.

From the results obtained in this series of experiments it is seen that after treatment with nitrous acid triamine can be quantitatively distilled. The diamine can be recovered by reducing the nitrosoamine formed and distilling.

In the instances where there was no di- or triamine present or when both were absent from the solutions used for analysis, a small reading was obtained on the burette. This is more apparent in the case of the diamine. If the oxides of nitrogen are not completely eliminated and should be distilled with the nitrosoamine, they are reduced to ammonia in the treatment with nascent hydrogen, giving higher results in the subsequent distillation of the diamine.

TABLE XI.

Determination of Di- and Triamine after Treatment with Nitrous Acid (Using 20 cc. of Saturated $NaNO_2$ and 10 cc. of Acetic Acid).

Sample contained.	Diamine (0.05 N).		Triamine (0.05 N).	
	Present.	Found.	Present.	Found.
	cc.	cc.	cc.	cc.
Dimethylamine	23.05	23.22	0.00	0.00
	23.55	23.50	0.00	0.00
	23.22	23.28	0.00	0.05
	23.17	23.22	0.00	0.00
	23.11	23.33	0.00	0.00
Trimethylamine	0.00	0.05	19.44	19.39
	0.00	0.06	19.39	19.44
	0.00	−0.05	19.44	19.44
	0.00	0.05	20.70	20.70
	0.00	Lost.	20.70	20.60
Diethylamine	17.30	17.20	0.00	0.30
	8.65	8.70	0.00	0.20
	8.65	8.65	0.00	0.20
	10.00	9.77	0.00	0.44
	5.00	5.00	0.00	−0.05
	5.00	5.05	0.00	0.00
Ethylamine	0.00	0.38	0.00	0.11
	0.00	0.27	0.00	0.11
	0.00	0.00	0.00	0.22
	0.00	0.22	0.00	0.22
"	0.00	0.10	0.00	0.15
	0.00	0.15	0.00	0.20
	0.00	0.15	0.00	0.15
	0.00	0.25	0.00	0.10
Dimethylamine and trimethylamine.	15.44	16.15	4.14	4.15
	11.58	12.35	8.28	8.25
	7.72	9.20	12.42	12.30
	3.86	3.90	16.56	16.10
	7.72	8.05	12.42	12.25
	3.86	4.10	16.56	16.72
	15.44	15.00	4.14	4.20
	11.58	11.25	8.28	8.35
	7.72	7.55	12.42	12.45
	3.86	4.05	16.56	16.50

Based upon the results obtained in the preliminary experiments, the following procedure, for the separation of ammonia and amines and their quantitative determination, was outlined. The method proposed depends upon the reaction of ammonia with oxide of mercury, in alkaline solution, and upon the reactions of the three classes of amines with nitrous acid and the subsequent reduction of the diaminenitrosoamine formed to diamine with nascent hydrogen. The method is applicable to the analysis of the end-products of the Kjeldahl digestion as well as to the analysis of the volatile alkaline materials obtained in the aeration method for the determination of ammonia in food and biological products. The following method was adopted.

Total Volatile Nitrogen.

This is usually known as the sum of the titration values composing the distillates. If it is to be determined, distil the combined distillates into 0.05 N acid and titrate the excess acid with 0.02 N alkali, using methyl red as the indicator.

Total Amines.

Take a volume of solution which contains an amount of total volatile nitrogen equivalent to from 40 to 60 cc. of 0.05 N acid. Transfer to a 500 cc. volumetric flask and fill to within 20 cc. of the mark; add 10 cc. of an alkaline mixture composed of equal parts of 20 per cent sodium hydroxide and 30 per cent sodium carbonate. Fill to the mark and add the yellow oxide of mercury reagent (0.1 gm. for each cc. 0.1 N equivalent to the total volatile nitrogen). Cover the flask with a black cloth and shake for 1 hour. After standing over night, force the solution through a cotton filter by a moderate air pressure. Measure accurately two 200 cc. portions and distil into 25 cc. of 0.05 N acid. Titrate the excess of acid using methyl red as the indicator. The acid used is equivalent to the amines present from which the total amines may be calculated.

Ammonia.—The difference between the total volatile nitrogen and the total amines is equivalent to the ammonia nitrogen.

Monoamines.—To the combined distillates obtained in the above separation of the total amines from ammonia, add 0.5 cc. of con-

centrated sulfuric acid and evaporate on the steam bath to 30 to 35 cc. Transfer to a 50 cc. volumetric flask, cool, and make up to the mark. Determine monoamine in 10 cc. in the Van Slyke amino nitrogen apparatus, using the mixing bulb of the large size apparatus and the measuring burette of the micro-apparatus. Allow the reaction in the deaminizing bulb to proceed for 30 minutes and shake the apparatus for 5 minutes. Calculate to percentage of total amines or to mg. of nitrogen as monoamine.

Triamines.—Transfer the remaining 40 cc. to a 200 cc. Erlenmeyer flask and rinse the 10 cc. pipette used for measuring the monoamine portion into this flask. Add 20 cc. of a saturated solution of sodium nitrite and 10 cc. of glacial acetic acid. Cover the flask with a small watch-glass, mix thoroughly, and allow it to stand for $\frac{3}{4}$ to 1 hour. Shake well to remove as much of the excess nitrogen gases as possible. Add a drop of phenolphthalein indicator and through a funnel which dips below the surface of the liquid in the flask, pour 30 cc. of a caustic soda solution made by dissolving 20 gm. of stick sodium hydroxide in 100 cc. of water. After a few minutes standing, mix carefully, making sure that the solution remains acid at all times, and adding a few drops of acetic acid if the solution begins to turn red. If all the solutions used are measured rather accurately in a graduate, the solution in the flask will remain acid after mixing. Allow the mixture to stand for several hours, if convenient over night, as determinations made with this length of time for the adjustment of the solution tend to be more accurate. Transfer to the distillation flask, add 15 to 20 cc. of the caustic soda solution, and distil into 25 cc. of 0.05 N sulfuric acid. Titrate back with 0.02 N alkali, using methyl red as the indicator. The acid used is equivalent to the triamine. Calculate to percentage of total amines or to mg. of nitrogen as triamine.

Diamines.—The diamines may be determined by difference. Or to the distillate obtained above add 10 cc. of concentrated hydrochloric acid and a few gm. of granulated zinc. As soon as a fair evolution of hydrogen has begun, place over a small flame and heat to boiling. Decant the solution from the remaining zinc and wash three times with 10 cc. portions of water, rinsing out the flask each time. Add a drop of phenolphthalein and about 10 gm. of stick sodium hydroxide and distil into 25 cc. of 0.05 N

sulfuric acid. Titrate back with 0.02 N alkali, with methyl red as the indicator. The acid used is equivalent to the sum of the diamine and triamine present. Calculate to per cent of total amines or to mg. of nitrogen as diamine.

TABLE XII.

Analysis of Known Mixtures Containing Ammonia and Amines.

Ammonia (0.05 N).		Monoamine (0.05 N).		Diamine (0.05 N).		Triamine (0.05 N).	
Present.	Found.	Present.	Found.	Present.	Found.	Present.	Found.
cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.
24.00	24.22	4.20	3.91	3.80	Lost.	20.60	20.56
24.00	24.22 24.10	4.20	4.01	3.80	3.56	20.60	20.69
24.00	24.10 24.22	4.20	4.01	3.80	3.56	20.60	20.63
24.00	23.85 25.10	4.20	3.91	3.80	3.50	20.60	20.63
23.90	23.87 23.87	4.20	4.03 4.03	1.95	2.03 1.88	20.70	20.78 20.78
23.90	23.87 23.87	4.20	4.20 4.01	1.95	2.03 1.88	20.70	20.78 20.78
24.20	23.90 24.02	2.10	Lost. "	3.90	Lost. 3.90	20.70	20.94 20.78
24.20	23.90 24.02	2.10	Lost. "	3.90	3.90 3.90	20.70	20.94 20.78

In Table XII the results of a series of analyses are given of solutions containing known amounts of ammonia and amines by the method as previously stated.

In Table XIII a series of determinations are given to show the accuracy of separation on applying the method to solutions of the amines when the total quantity present approximates 2 cc. of 0.05 N in strength. The results of duplicate determinations are given.

In order to test further the accuracy of the method, unknown solutions were prepared and submitted to analysis. The results obtained are given in Table XIV.

The method requires experience in the use of the Van Slyke amino-acid apparatus, otherwise its operation is quite simple. The points in the manipulation of the method, which it is important to bear in mind to secure uniform results, are set forth in the following summary.

TABLE XIII.

Separation of Amines When the Total Quantity Present Approximates 2 Cc. 0.05 N.

Solution.	Monoamine.		Diamine.		Triamine.		Total amines.	
	Present.	Found.	Present.	Found.	Present.	Found.	Present.	Found.
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
I	0.8	0.8	0.0	0.2	0.7	0.8	1.5	1.8
		0.9		0.0		0.9		1.8
II	0.4	0.5	0.3	0.5	0.7	0.8	1.4	1.8
		0.4		0.3		0.9		1.6
III	0.8	0.7	0.3	0.5	0.4	0.4	1.5	1.6
		0.7		0.3		0.6		1.6
IV	0.4	0.6	0.0	0.1	1.1	1.3	1.5	2.0
		0.4		0.0		1.2		1.6
V	0.0	0.1	0.3	Lost.	1.1	1.3	1.4	1.4
		0.0		0.3		1.2		1.5

1. The caustic soda solutions were prepared by dissolving 20 gm. of stick sodium hydroxide in 100 cc. of water.

2. In shaking with mercuric oxide the flasks must be wrapped in a black cloth because the precipitate, containing a combination of ammonia and mercuric oxide, becomes black on exposure to light, showing decomposition. Several attempts were made to find the composition of the precipitate but the salt decomposed so readily that this was not accomplished.

3. All the solutions used in decomposing monoamine and changing diamine to nitrosoamine must be measured accurately with a graduate in order that the solutions may remain acid when mixed.

4. If the oxides of nitrogen are distilled over with nitrosoamine and triamine they will affect the results in two ways: (*a*) the triamine will be low on account of the added amount of acid to be titrated, (*b*) in the subsequent treatment with nascent hydrogen, the oxides of nitrogen will be reduced to form ammonia and make the determination of diamine higher. The color of the

TABLE XIV.

Analysis of Unknown Solutions.

	Present (0.05 N).	Found (0.05 N).	Nitrogen found.
By J. B. W.			
	cc.	*cc.*	*mg.*
Total volatile nitrogen	50.23	50.30	35.21
Ammonia nitrogen	9.30	9.42	6.59
Monoamine "	10.58	10.12	7.08
Diamine "	9.65	10.20* / 9.75*	7.14
Triamine "	20.70	21.01	14.70
Total amine " (determined)	40.93	41.33	28.92
" " " (by difference)	40.93	40.88	28.62
By F. C. W.			
Total volatile nitrogen	57.20	57.00	39.90
Ammonia nitrogen	5.70	6.45	4.52
Monoamine "	21.15	21.30	14.90
Diamine "	9.65	9.70	6.90
Triamine "	20.70	19.20	13.70
Total amine " (determined)	51.50	50.20	35.50
" " " (by difference)	51.50	50.55	35.38

* By difference.

methyl red is destroyed when the oxides of nitrogen come over in the distillation.

5. To prevent oxides of nitrogen from being carried over in the distillation, (*a*) agitate the solution well to get rid as much as possible of the gaseous oxides of nitrogen before adding 30 cc. of the caustic soda solution; (*b*) in transferring to the distillation of flask pour the solution down the side of it, to prevent bubbles of the gas from forming; (*c*) allow the solution to stand for a few

minutes before adding the second amount of alkali; (*d*) just before making alkaline, blow out the air above the solution.

6. Prevent loss in distillation by using on the end of the condenser an adapter which has been closed off and has had a number of small holes made with a platinum wire in the sides and bottom.

7. Prevent loss of triamine by adding caustic soda solution through a funnel which dips below the surface of the liquid in the flask. When added in this manner the heavy soda solution sinks to the bottom of the flask and neutralizes the solution from below, leaving the exposed surface with an acid reaction.

8. Prevent loss of triamine and nitrosoamine by reducing the volume of the solution to as little as possible in the distillation. *Do not* allow sodium nitrite to crystallize out, for the liquid may suddenly solidify and under such conditions will decompose at once with the formation of alkaline materials.

9. To insure complete distillation of di- and triamines after reduction, carry the distillation until only a few cc. of liquid are left in the flask. At this point there is no danger of decomposition.

10. Solid caustic soda is used for this distillation to keep down the volume of liquid and in order to prevent loss from the hot solution by again neutralizing it from below.

THE INHIBITION OF FOAMING.

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The troublesome foaming encountered when air is blown through solutions containing proteins, soaps, etc., or when such solutions are subjected to low pressures, must be dealt with in many analytical and other operations now in use in biochemical laboratories. Since the most effective agents for preventing this foaming (*i.e.* for accelerating the rupture of the bubbles) are not now readily accessible to many who have occasion to use them, an attempt has been made, in this paper, to outline briefly the manner of action of such agents, and to determine their essential properties. From the information thus obtained, it has been possible to find an efficient foam inhibitor, which can be prepared with ease from available materials.

It can readily be shown that this foaming cannot be adequately prevented by an organic liquid which is completely dissolved. The presence of undissolved particles of the liquid must therefore be regarded as essential.[1] Something may be learned of the factors involved in the inhibition of foaming from the behavior of drops of various liquids upon a clean water surface, a matter which has been investigated especially by Hardy.[2]

When a soap bubble comes into contact with a drop of such a liquid as isoamyl alcohol, under the proper conditions, a film of

[1] Hence the importance (*e.g.* in the urease method) of increasing the number of such particles by thoroughly shaking the mixture after adding the inhibiting agent, and *before* starting the air current.

[2] Hardy, W. B., *Proc. Roy. Soc., Series A*, 1912, lxxxvi, 610; 1913, lxxxviii, 303, 313. See also Pockels, A., *Physik. Z.*, 1916, xvii, 142; abstracted in *Chem. Abst.*, 1917, xi, 2067. Rayleigh, *Phil. Mag.*, 1918, xxxv, 157.

the liquid may be seen beginning to spread over the surface of the bubble, and the bubble presently bursts. A region of diminished surface tension is produced beyond the visible edge of the spreading drop of isoamyl alcohol. The rupture of the bubble may be accounted for by the existence of two forces, opposite in sense, acting upon this region at the periphery of the drop; *viz.*, (1) the tension of the uncontaminated part of the soap film itself, directed outwards, and (2) the tension of the double surface,[3] acting inwards. The rapidity of rupture must depend upon the magnitudes of these two forces, among other factors, and should be greater, other things being equal, the greater the tension of the double surface, and the greater the decrease in surface tension beyond the edge of the drop, which depends not only upon the surface activity of the liquid, but also upon its volatility.[4, 5]

The chief properties which contribute to the efficiency of an organic liquid as a foam inhibitor may, from the above discussion, be enumerated as follows: high surface activity, high surface tension, high interfacial tension, low solubility, low volatility.[6] The conditions imposed are not so complex as they may at first sight appear, since they are to a certain extent dependent upon the same fundamental properties. Solubility and volatility, for example, are, within limits, inversely related to interfacial tension.[7] Further, among related substances, solubility and surface activity are inversely related.[8, 9, 10]

The surface tension data available[7, 11] apply, for the most part,

[3] *I.e.*, the tension of the alcohol-air surface, plus that of the interface between alcohol and soap solution.

[4] Hardy, *Proc. Roy. Soc., Series A*, 1913, lxxxviii, 315–316.

[5] No account has been taken here of the adsorption of colloidal material by dispersed particles of the organic liquid, since this is apparently of secondary importance.

[6] This statement is true, of course, only within limits; *e.g.*, an appreciable degree of solubility is necessary.

[7] Harkins, W. D., Brown, F. E., and Davies, E. C. H., *J. Am. Chem. Soc.*, 1917, xxxix, 354. Harkins, W. D., Davies, E. C. H., and Clark, G. L., *ibid.*, 541.

[8] Traube, J., *Ber. chem. Ges.*, 1884, xvii, 2294; 1909, xlii, 86; *Arch. ges. Physiol.*, 1904, cv, 541.

[9] Worley, R. P., *J. Chem. Soc.*, 1914, cv, 260, 273.

[10] Tinker, F., *Phil. Mag.*, 1916, xxxii, 295.

[11] Langmuir, I., *J. Am. Chem. Soc.*, 1917, xxxix, 1892.

only to pure organic liquids and their pure aqueous solutions; their use for the present purpose should nevertheless make it possible to avoid much unnecessary work in preparing and testing out materials. Among those substances for which data exist, the above conditions are best met by esters of relatively high molecular weight. An efficient foam inhibitor has accordingly now been found in isoamyl isovalerate (or a mixture of this substance with isoamyl alcohol). Two methods of preparing this reagent are described below.

Preparation of the Reagent.

First Method.—To 35 cc. of commercial isovaleric acid add 4 gm. of anhydrous calcium chloride. Shake the mixture until it has separated into two layers and most of the calcium chloride has dissolved. The greater part of the water is in this way removed. Decant off the upper layer, and transfer 28 cc. of this to an Erlenmeyer flask, of about 200 cc. capacity, containing a few glass beads. Add 30 cc. of isoamyl alcohol, and 1 cc. of concentrated sulfuric acid. Connect the flask with a reflux condenser, and boil for 1 hour over a wire gauze. Cool, transfer to a separatory funnel, and draw off the lower layer. To the upper layer add water, and shake the mixture thoroughly with solid sodium carbonate until a sample of the aqueous layer, drawn off through the stem, is found to be alkaline to phenolphthalein. Separate again, and remove the excess alkali by washing with water. After drawing off the final wash water (which should not be alkaline to phenolphthalein), filter the residual liquid. The filtrate is then ready for use. It will, however, keep better if dried with calcium chloride and distilled.

Second Method.—[12, 13, 14] Dissolve 50 gm. of potassium dichromate in 200 cc. of boiling water. Cool under the tap, with constant shaking. The bulk of the dichromate will separate out in small crystals. Add 50 cc. of isoamyl alcohol. While the mixture is kept constantly agitated under the tap, now add 70 cc. of concentrated sulfuric acid at such a rate that the temperature of the mixture does not rise above 30°. Let stand at room temperature over night. Separate the two layers, transferring the upper one to a 500 cc. distilling flask. Add 200 cc. of water, and distill through a water-cooled condenser until all of the ester has come over. Neutralize the distillate with sodium carbonate, wash with water, etc., as in the first method.

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[12] Kopp, H., *Ann. Chem.*, 1855, xciv, 297.

[13] Pierre, I., and Puchot, E., *Compt. rend. Acad.*, 1869, lxix, 266; *Ann. chim. et phys.*, 1871, xxii, 344; *Ann. Chem.*, 1870, cliii, 260; 1872, clxiii, 289.

[14] This second reagent has the disadvantage, for certain purposes, of containing some isovaleraldehyde, which can, however, be removed by shaking with bisulfite solution.